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XCIII. THE NATURE OF THE BLOOD-SUGAR.

By LEWIS BLAND WINTER (*Grocers' Company Research Scholar*).

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(*Received April 8th, 1930.*)

Isolation of the blood-sugar has been seldom attempted. Hédon [1898] worked with the blood of depancreatised dogs. The proteins were separated by dialysis for several days at low temperature, and the solution containing the sugar was evaporated to a syrup in open vessels at 60–70°. Partial purification was effected by precipitation with neutral lead acetate; the filtrate after removal of lead was evaporated. The solid precipitated from the syrup by addition of ether gave glucosazone on heating with phenylhydrazine. The reducing power determined by titration with Fehling's solution showed a considerably greater glucose content than that deduced from the optical activity. Similar results were obtained with the blood-sugar of the horse; the glucose content estimated by the polarimeter was rather more than half that found by titration. Hédon found substantial agreement between the two methods in the case of sugar prepared from urine in diabetes, and concluded that the urinary sugar differed from that of the blood, undergoing some modification at the moment of its secretion. Hanriot [1898] used horse blood, which was deproteinised by boiling with sodium sulphate; the filtrate was concentrated and the syrup purified by repeatedly dissolving in methyl alcohol and precipitating with ether. A dry powder was obtained, and when the sugar was estimated by Fehling's solution or polarimetrically there was good agreement. The sugar could be converted into glucosazone or parachloralose. Neither Hédon nor Hanriot records analyses or melting-points of the substances isolated by them, nor was the sugar pure enough to show mutarotation. The sugar content given by the polarimeter was deduced from the equilibrium value of α- and β-glucose. This value was also used in the calculation of the sugar content of blood-filtrates by Winter and Smith [1923] and compared with that found by estimation of the copper-reducing power. It was found that blood-filtrates from normal men and animals showed a marked discrepancy between the figures obtained by the two methods, but that this difference disappeared in the course of some days. On the other hand, in diabetes the values agreed from the outset, and it was suggested that the sugar was of a different type in the two cases. Subsequently, Lundsgaard and Holbøll [1925] came to a similar conclusion. Denis and Hume [1924] repeated the experiments of Winter and Smith [1923] and concluded that the changes in optical activity on keeping the sugar solutions were due to contamination

with micro-organisms; the changes recorded by them were, however, in the opposite direction. In all the above experiments observations were made on sugar solutions contaminated with varying amounts of other substances. The object of the present work was to separate the sugar from normal blood in such a way as to enable it to be identified with certainty.

Experimental.

The blood of the rabbit has been used throughout. The animal having been killed by a blow on the head, the throat is cut and the blood received into a shallow basin. The blood is defibrinated by whipping and the volume measured. The proteins are precipitated with the Folin-Wu reagents, seven volumes of water being used for dilution. After vigorous shaking the mixture is allowed to stand for an hour and then filtered through papers which have been very thoroughly washed with water. When filtration is complete the precipitate is washed with a little cold water. The total volume of filtrate measures about 800 cc. for 100 cc. blood. Concentration, which is carried out *in vacuo* at 35–40° until the volume is rather more than half the original volume of blood, should be completed in 2–3 hours. The concentrated solution is transferred to a flask, and the distilling flask washed out with saturated lead acetate, 0·5 cc. per cc. blood. The lead acetate is added to the sugar solution. After shaking, the mixture is allowed to stand for an hour. The bulky precipitate is centrifuged, and washed with small amounts of water. The clear solution is cooled in ice, and made alkaline with strong ammonia. The precipitate is allowed to settle for a few hours, being kept cold, and then separated by the centrifuge. A fifth of the previous quantity of lead acetate is added to the fluid, and a further small amount of ammonia. The second precipitate is centrifuged in the same cups as contain the first. The precipitate is stirred up with 80 % alcohol until all lumps have disappeared and then spun down. The washing is repeated. The cups and precipitate are dried in a vacuum over sulphuric acid. The lead compound may be kept for some time in the dry state if convenient. The dry precipitate is weighed and replaced in a centrifuge cup. For each gram 2 cc. 2*N* sulphuric acid are added, and the precipitate is disintegrated with a glass rod; almost continuous stirring for 10 minutes is advisable for complete decomposition. If not acid to litmus further acid is cautiously added, with stirring, until a test sample is distinctly acid. The fluid is separated in the centrifuge and transferred to a measuring cylinder. The precipitate is stirred with a small amount of water, separated, and the washing added to the first solution. The volume is noted. The solution is transferred to a small flask, and 4 volumes of strong alcohol are added gradually, shaking between each addition. The lead precipitate is washed twice with 5 cc. of 80 % alcohol, and the washings are added to the flask. It is advisable to allow a day in the cold chamber for complete precipitation; haste at this stage is liable to cause difficulty later. The solution of the sugar in alcohol is centrifuged, and the fluid, which

should be quite clear, is separated. It is better not to wash the precipitate as there is a tendency to form a colloidal suspension. The solution is cooled in ice, and a slight excess of 10 % baryta in methyl alcohol is added. After standing for an hour in ice, the barium compound is separated in the centrifuge and washed twice with cold 80 % alcohol. The precipitate is suspended in 80–100 cc. of 80 % alcohol, warmed to 40°, and decomposed with a rapid stream of CO_2 for 2 hours. After 24 hours in the cold room the carbonate is filtered off, and the solution completely evaporated under reduced pressure. To the distilling flask 15 cc. of 80 % alcohol are added, a trace of insoluble matter is filtered off, and the solution allowed to evaporate slowly in a small basin over sulphuric acid. There is left a colourless syrup which begins to crystallise on stirring. Very soon there is a hard crystalline mass which is removed, rapidly washed with alcohol, and dried. The yield from 100 cc. blood averages 36 mg. To obtain pure α-glucose the crude product is twice recrystallised from dry methyl alcohol, best by allowing the solvent to evaporate very slowly at room temperature; the sugar crystallises in fine needles which are washed with alcohol and dried in a vacuum over sulphuric acid.

Melting point. 145–146° (uncorr.).

Analysis. (Micro-analyses by Dr Ing. A. Schoeller, Berlin.)

C 39·87, 39·73 %; H 6·71, 6·64 %.

$C_6H_{12}O_6$ requires C 40·00 %; H 6·66 %.

Optical activity. All readings were taken in a 1 dm. tube of 4 mm. bore. Only 1·5 cc. of fluid are required, and it is possible with this tube to obtain as great a degree of accuracy as when a tube of standard bore is used. The polarimeter was a three field instrument; a half shadow angle of 1° was used. All readings agreed within 0·02°. The substance shows mutarotation in water and dry methyl alcohol; the rate of change in the latter solvent is slow. 17·4 mg. were dissolved in 1·6010 g. water. $c = 1{\cdot}08$.

Time (hrs.)	0·08	1	1·5	3·1	9·0	18
α	+1·36	1·00	0·90	0·80	0·66	0·66°
$[\alpha]^{14°}_{5461}$	+125	92	83	74	61	61°

8·4 mg. were dissolved in 1·6155 g. methyl alcohol (*d.* 0·799). $c = 0{\cdot}41$.

Time (hrs.)	0·08	7	22	44
α	+0·56	0·52	0·50	0·46°
$[\alpha]^{14°}_{5461}$	+136	126	121	112°

Molecular weight. Determinations were carried out by Barger's method. Methyl alcohol containing 25 % of water was used as solvent, and urea as standard. 10·5 mg. sugar were dissolved in 0·522 g. methyl alcohol of *d.* 0·878, giving a concentration of 17·6 g. per litre. This solution was sealed in capillary tubes with the urea solutions. Tube 1 contained 0·09 *M*, tube 2, 0·1 *M*, tube 3, 0·11 *M* urea. After reading, the tubes were left for 15 hours at room temperature. In tube 1 the urea was hypotonic, in tube 3 hypertonic. In tube 2 no increase or decrease took place in the size of the drops, giving a molecular weight of 176. In another experiment a value of 188 was obtained.

Preparation of the phenylosazone. 85 mg. of the sugar were dissolved in 4·5 cc. of water and 0·25 g. phenylhydrazine hydrochloride and 0·55 g. sodium acetate were added. The mixture was heated on a boiling water-bath for half an hour and allowed to cool slowly. The crystals were separated, washed with water, and recrystallised from dilute alcohol. Yield 32 mg. Rapidly heated the melting-point was 208–210°, unchanged when the substance was intimately mixed with a known sample of glucosazone. The optical activity of the product was observed when dissolved in a mixture of 6 parts of pyridine and 4 parts of absolute alcohol. 8·7 mg. in 1·5 g. solvent (*d.* 0·873). $c = 0{\cdot}50$.

Time (hrs.)	0·25	22
α	−0·40	0·22°
$[\alpha]^{13°}_{5461}$	−80	40°

The following figures were obtained on analysis:

C 59·76, 59·92 %; H 6·28, 6·18 %; N 15·57, 15·54 %.

$C_6H_{10}O_4(N : HN.C_6H_5)_2$ requires C 60·33, H 6·14, N 15·64 %.

Preparation of the β-penta-acetate. A mixture of 0·14 g. of the sugar and 0·07 g. of freshly fused sodium acetate was heated with 0·7 cc. of acetic anhydride in a small tube fitted with a reflux condenser. After 2 hours at 100° 4 cc. of cold water were added. The oil which precipitated rapidly crystallised on stirring. The product was washed with several changes of cold water, and three times recrystallised from 90 % ethyl alcohol, in order to remove as much of the α-compound as possible. Yield 34 mg. M.P. 124–125°. $[\alpha]^{13°}_{5461} = +5{\cdot}5°$ (in chloroform).

C 48·84, 49·54 %; H 5·77, 5·90 %.

$C_6H_7O_6(OC.CH_3)_5$ requires C 49·23; H 5·64 %.

From a consideration of the experiments described it seems clear that the blood-sugar consists largely of glucopyranose. It has not been possible during the present work to obtain definite evidence as to the presence of any other sugar. The accepted figure for the concentration of reducing substances in normal blood varies from 0·08 to 0·1 %, and the yield of crystalline glucose obtained, taking into account the losses attendant on the various precipitations, does not suggest that more than traces of other sugars can be present; this question is being considered in connection with the metabolism of lactose and will be dealt with in a later communication. In numerous experiments the mother-liquor has been worked up further after crystallisation of the glucose; the syrup obtained has a strongly positive rotation, and gives glucosazone on treatment with phenylhydrazine. The source of the fasting blood-sugar is liver-glycogen. Recent views as to the constitution of this polysaccharide agree that glycogen consists of a conjugated chain of α-glucose units [Haworth, Hirst and Webb, 1929]. When α-glucose is liberated by the action of the liver diastase *in vivo* it seems likely that under the influence of body temperature and blood alkalinity a mixture of the α- and β-stereoisomers will be rapidly established, unless there are some factors involved of which

we have no knowledge. Allott [1928] working with rabbits, found no definite difference between the blood-sugar curves after injection of either isomer, or of the equilibrium mixture. The explanation of the low values obtained when the sugar in deproteinised blood-filtrates is estimated by the polarimeter is not yet clear. It has been suggested [Holden, 1926] that glutathione, or ergothioneine [Sjollema, 1927] may be responsible, but the experiments with rabbits after insulin [Winter and Smith, 1924] are difficult to reconcile with this view. When the blood-sugar was removed under the action of insulin and the blood-filtrates examined polarimetrically, a small positive value was always obtained. If any strongly laevo-rotatory substances pass into the Folin-Wu filtrates they should become apparent when the glucose is removed, unless insulin has the effect of diminishing their concentration *pari passu* with the blood-sugar.

SUMMARY.

The blood-sugar of the rabbit has been isolated as α-glucopyranose.

I wish to thank Professor W. N. Haworth for his interest and advice. Part of the expense of this work was defrayed by a grant from the Government Grant Committee of the Royal Society.

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XCIV. THE DISSOCIATION OF CALCIUM CITRATE.

By WALDO JACKSON WILEY.

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(*Received May 9th, 1930.*)

It is often stated that the addition of a citrate to milk prevents clotting by rennin because an un-ionised calcium citrate is formed, thus removing the calcium ions from solution, these ions being considered essential for the clotting process. The prevention of the clotting of blood by citrates is explained in the same way.

It is not the purpose of this paper to discuss the clotting phenomenon, but to record some measurements of the dissociation of calcium citrate. However, it must be pointed out that there is some confusion on the subject. A typical analysis of milk-serum [Van Slyke and Bosworth, 1915] shows a concentration of 0·465 g. of calcium per litre and 2·37 g. of citric acid. The citrate is present in a larger proportion than is necessary for equivalence with the calcium, so if un-ionised tricalcium citrate were formed there would be no calcium ions present. Analyses in this laboratory have shown the above figures to be typical of milks which clot normally with rennin. If the theory mentioned in the first paragraph were quite correct, most milks would not clot, for there is normally enough citrate present to prevent this.

Shear and Kramer [1928] and Shear, Kramer and Resnikoff [1929] give results of conductivity titrations of calcium salts with sodium citrate and claim that the results give "direct evidence for the binding of calcium ions by sodium citrate in some kind of soluble complex." They obtained a minimum conductivity when the added sodium citrate was approximately equivalent to the calcium present. As they adjusted the p_H of their solutions with uncertain proportions of acid and alkali, it is impossible to make an estimate of the conductivity of the solution due to any calcium ions present. The only safe deduction from their experiments seems to be that calcium citrate is less ionised than calcium chloride or sodium citrate at the same dilution. Stewart and Percival [1928] state that they have found by conductivity measurements that calcium citrate is almost undissociated in solution, but they give no particulars. It was, therefore, decided to publish the following measurements of the conductivity of simple calcium citrate solutions.

Method and apparatus.

Conductivities were measured by the usual method, using a metre bridge and post-office box with a small induction coil as source of current. The con-

ductivity cell was of 120 cc. capacity and contained electrodes of about 1 cm.^{2} area placed 1 cm. apart. The cell constant was determined by the measurement of the conductivity of $N/10$ potassium chloride at 18° [Reilly, Rae and Wheeler, 1926].

Conductivity water was obtained by redistilling water obtained from a still of the form described by Golding [1906], rejecting the first and last portions of the distillate. It was allowed to come to equilibrium with the atmosphere and usually had a conductivity of about 2×10^{-6} mhos. This was deducted from the values obtained for the conductivity of the solutions measured.

Calcium citrate solution.

This was prepared by mixing concentrated solutions of A.R. calcium chloride and sodium citrate in equivalent proportions. Calcium citrate rapidly precipitated in the high concentration used and was washed repeatedly with distilled water by decantation until 500 cc. of the wash water, when concentrated to 20 cc., gave no reaction for chlorides. The precipitate was then washed a further ten times with conductivity water and finally allowed to stand 2 days in contact with conductivity water in order to prepare the solution for conductivity measurements. This solution was obtained by careful decantation from the solid calcium citrate. All this manipulation was done in one Pyrex flask and the solution stored in Pyrex glass. No attempt was made to obtain a saturated solution of calcium citrate but the difficulties encountered by Shear and Kramer in dissolving calcium citrate were avoided by this procedure.

150 cc. of the solution evaporated to dryness and dried to constant weight at 150° gave 0·1050 g. of total solids containing 0·0251 g. Ca; $Ca_3(C_6H_5O_7)_2$ requires 0·0253 g.

Table I gives results of the measurements of conductivity of different dilutions of this solution at 18°.

Table I.

Temperature 18°.

Normality of $Ca_3(C_6H_5O_7)_2$ solution	Specific conductivity (mhos.)	Equivalent conductivity	Degree of dissociation, α
0·00846	0·000176	20·8	0·188
0·00508	0·000118	23·2	0·209
0·00305	0·0000758	24·8	0·224
0·00183	0·0000498	27·2	0·245
0·00110	0·0000336	30·5	0·275

The degree of dissociation, α, was calculated by taking the values for the mobilities of the Ca^{++} and citrate$\equiv$ ions at 18° as 51 and 60 respectively.

On the assumption that we are simply dealing with the dissociation of $Ca_3(C_6H_5O_7)_2$ it is seen that the dissociation at these high dilutions is small, but by no means negligible (cf. the slight dissociation found by Riley and Fisher [1929] for some metal malonates).

Titration of citric acid with calcium hydroxide.

In order to confirm the above figures and to obtain data for more concentrated solutions, conductivity titrations of citric acid solutions with calcium hydroxide solution were made. As there were no foreign ions present in these solutions it was possible to calculate the dissociation of the calcium citrate formed in the titration.

The citric acid was recrystallised from A.R. grade citric acid, and the calcium hydroxide solution was prepared from lime obtained by heating Kahlbaum's chemically pure calcium carbonate. All solutions were made up using conductivity water.

The results of a titration of 100 cc. of 0·0104 *N* citric acid with 0·0455 *N* calcium hydroxide at 18° are shown in Fig. 1. An electrometric titration of the same solutions is also graphed. This was made by using the quinhydrone

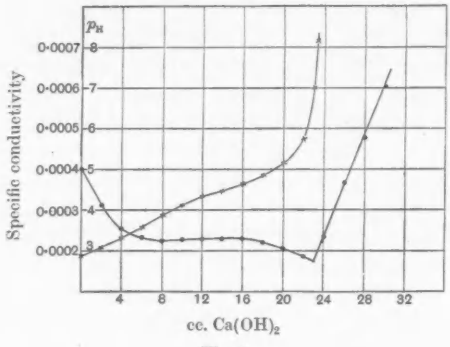

Fig. 1.

•——• Sp. conductivity. ×——× p_H.

electrode for p_H measurements. There is a sharp break in the conductivity curve when the calcium hydroxide is present in the proportion necessary for the formation of tricalcium citrate and this end-point is confirmed by the electrometric titration. The volume of calcium hydroxide solution added at the end-point and the conductivity of the resultant calcium citrate solution are readily obtained from the curve, and so the normality of the solution with respect to calcium citrate, and the dissociation, may be calculated.

Similar titrations were made of various concentrations of citric acid. The most concentrated solution used was 0·0414 *N* at the end-point. This is comparable to the usual concentration of "citric acid" in milk (about 2 g. per litre). At this concentration the solution is super-saturated. It was found that the conductivity obtained immediately after adding the equivalent proportion of calcium hydroxide did not vary over a period of 5 hours. After standing for about a week calcium citrate had precipitated but the change in conductivity over this period was not investigated.

Table II gives the results of these titrations performed at different dilutions.

Table II.

Temperature 18°.

Normality of $Ca_3(C_6H_5O_7)_2$ at end-point	Specific conductivity at end-point (mhos.)	Equivalent conductivity	Degree of dissociation, α
0·0414	0·000672	16·3	0·147
0·0293	0·000496	16·9	0·152
0·0134	0·000248	18·5	0·166
0·00844	0·000170	20·2	0·183
0·00425	0·000100	23·5	0·212
0·00214	0·000055	25·7	0·232

The values obtained for the degree of dissociation by the two methods are shown in Fig. 2, where they are plotted against normality of the solution.

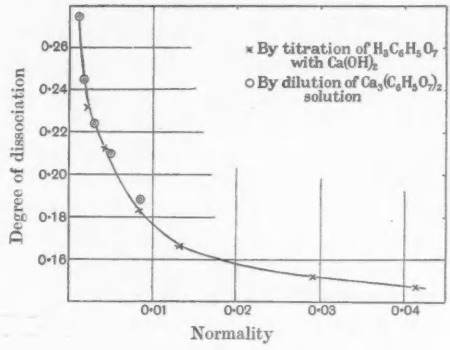

Fig. 2.

There is close agreement between the values. Of course the numerical values obtained for the degree of dissociation could not be transferred directly to such solutions as milk and blood. The effect of ionic concentration on the dissociation of such a substance as calcium citrate is uncertain. However, the figures give some idea of the order of magnitude of the dissociation.

Summary.

The dissociation of tricalcium citrate in aqueous solution at 18° has been studied, and values for the degree of dissociation are recorded.

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XCV. OBSERVATIONS ON A DIATOM (*NITZSCHIA CLOSTERIUM* W.SM.) AS A SOURCE OF VITAMIN A.

By BASHIR AHMAD.

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(*Received May 16th, 1930.*)

Introduction.

In 1922 Jameson, Drummond and Coward [1922] studied *Nitzschia closterium*, a typical unicellular marine diatom, and showed that it possessed considerable growth-promoting activity. At that time the fat-soluble vitamins A and D were not differentiated, and these tests, therefore, did not preclude the possibility of the observed growth being due to a factor other than vitamin A. This possibility was indirectly supported by the observation of Drummond and Watson [1922] that the oil from this organism failed to give a positive colour reaction with strong sulphuric acid which Rosenheim and Drummond [1920] had previously shown to be associated with vitamin A. On the other hand, in view of the experiments of Leigh-Clare [1927] it seemed improbable that the growth-promoting activity could be ascribed to vitamin D.

Recently the specificity of the colour reactions of vitamin A with $AsCl_3$ and $SbCl_3$, described by Rosenheim and Drummond [1925] and by Carr and Price [1926], in support of which a weight of evidence has accumulated during the last few years, has been questioned by various investigators. The discrepancies have been satisfactorily explained by Drummond and Morton [1929] and by Ahmad and Drummond [1930], but the controversy recalled the earlier observations of Drummond and Watson [1922] regarding the oil from *Nitzschia closterium* which, though apparently a potent source of vitamin A, did not give the colour reaction. It was decided, therefore, to repeat the experiments, and also get a more quantitative estimate of the activity of this organism in the light of our present knowledge of the subject.

Experimental.

Following mainly the technique of Allen and Nelson [1910], large quantities of the organism were grown from a pure culture obtained by the courtesy of Dr E. J. Allen, F.R.S., Director of the Marine Biological Laboratory, Plymouth. 100 cc. of sea water, to which Miquel's nutrient solutions, as modified by Allen, had been added, were placed into each of a large number of conical

Pyrex flasks of 250 cc. capacity, plugged with cotton-wool and sterilised in steam at 95° for 20 minutes on three successive days. Each flask was then inoculated with 0·01 cc. of the pure culture and placed in front of a window in diffused daylight. The bottoms of the flasks were soon covered with a brown growth of the organism.

A large quantity of the organism being required, cultures and subcultures were continued for a period extending over a year. Cultures usually stopped growing after 4–6 weeks. During the winter months when the daylight was poor the growth was scanty.

Four to six weeks-old cultures were filtered on a tared filter, dried in a vacuum desiccator and weighed. They were stored in a desiccator, which had been re-evacuated after the air had been replaced by nitrogen, in a cool, dark cupboard. The oil was extracted with redistilled ether by continuous extraction in Soxhlet's apparatus. Nitrogen was passed in during the extraction and other precautions were taken to prevent oxidation.

The results of culture experiments are summarised in Table I.

Table I.

Crop	Time of year	Vol. of the culture sol. (litres)	Dry wt. of organism (g.)	Quantity of oil obtained (g.)
1	Apr. and May	3·7	3·55	0·103
2	June, July and Aug.	12·5	11·74	—
3 (*a*)	Sept. and Oct.	5·5	5·53	0·156
(*b*)	Sept. and Oct.	3·0	1·10	—
4	Oct., Nov. and Dec.	15·5	9·075	0·374
5	Dec., Jan. and Feb.	15·0	2·765	

Under satisfactory conditions of growth approximately 1 g. of the dry organism was obtained from each litre of a month-old culture. During the dark winter days the yield fell even below 0·2 g. per litre. The amount of the oil in the dry organism was, however, fairly constant and varied between 2·8 and 3·1 %.

The oil is extremely rich in natural plant pigments, chlorophyll, xanthophyll, carotene, and fucoxanthin, the characteristic pigment of the brown algae. It gave a strong blue colour reaction with $SbCl_3$. Drummond and Watson failed to get the colour reaction with H_2SO_4, probably because they had rather a small amount of the material available, and because with this reagent the test is not very delicate.

Table II shows the yellow and blue colour units given by 10 % solutions in a 1 cm. cell and examined in the Rosenheim-Schuster colorimeter. In the case of blue units the figures refer to 0·2 cc. of 10 % oil mixed with 2 cc. of $SbCl_3$ reagent, and examined after 30 seconds. The technique of Carr and Price [1926] was mainly followed, though 10 % values were obtained from an intensity-dilution curve as explained in our previous communication [Ahmad and Drummond, 1930].

The pigment of *Nitzschia closterium* interferes with the application of the $SbCl_3$ reaction. Attempts have been made to obtain the non-saponifiable fraction of the oil in order to remove chlorophyll, but sufficient material was not available. For this reason the measurement of the blue colour must be regarded as approximate.

Table II.

Crop	Dilution %	Colour units 1 cm. cell	Blue units with $SbCl_3$ reagent
1	10	3,000 yellow 429 red	57·1 blue 52·4 yellow
3 (*a*)	10	10,000 yellow 830 red	100–150 blue 100–120 yellow
4, 5	10	10,000 yellow 800 red	100–150 blue 100–120 yellow

The technique of the biological test was that usually employed in this laboratory [see Drummond and Morton, 1929]. The basal diet consisted of purified caseinogen, rice starch and salt mixture without the addition of any extraneous fat. Vitamin D was supplied as irradiated ergosterol dissolved in medicinal liquid paraffin, and 0·01 mg. was given twice a week to each rat. Ethyl laurate was used as a diluent for the oil. Its satisfactory nature has been shown by Drummond, Ahmad and Morton [1930].

For feeding, the filter-paper with the organism was broken up into small pieces, mixed with a small quantity of water and the basal diet, and worked up into a dough. This was given to the rats in aliquot portions on small porcelain dishes. They devoured it readily.

The results of the biological tests are shown in Table III, and Figs. 1–3 show some representative curves.

Table III.

No. and sex of rat	Dose and its nature	Total days of test	Total difference in wt. (g.)	Rate of growth per week (g.)
27 ♂	0·2 mg. oil	16	− 3	− 1·3
26 ♀	0·4 mg. oil	16	0	0
28 ♂	1·0 mg. oil	16	− 11	− 4·8
69 ♂	0·35 g. organism	21	+ 45	+ 15·0
70 ♀	0·35 g. organism	21	+ 31	+ 10·0
72 ♀	0·1 g. organism	21	+ 27	+ 9·0
95 ♀	5·0 mg. oil	14	+ 13	+ 6·5
362 ♀	1 mg. oil	7	0	0
	5 mg. oil	14	+ 15	+ 7·5
385 ♂	2 mg. oil	11	− 5	− 3·2
	0·15 g. extracted organism	7	− 3	− 3·0
	0·15 g. extracted organism plus 5 mg. oil	14	+ 30	+ 15·0
388 ♀	3 mg. oil	11	+ 2	+ 1·2
	5 mg. oil	14	+ 15	+ 7·5

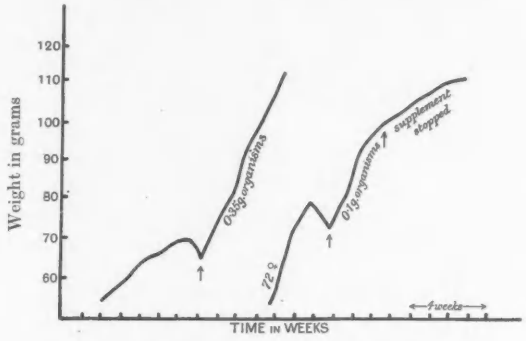

Fig. 1. Administration of dried organism.

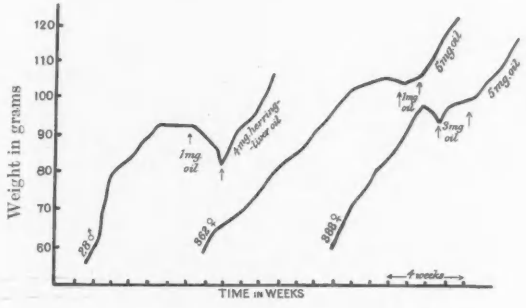

Fig. 2. Administration of oil.

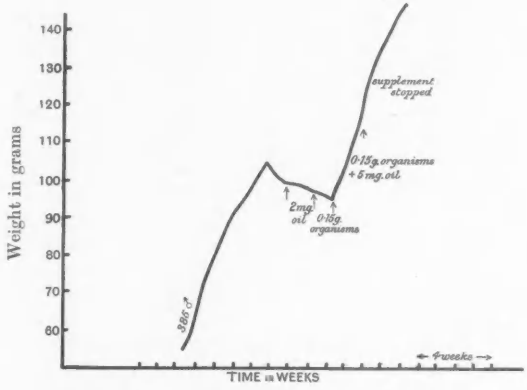

Fig. 3. Administration of extracted organism plus oil.

Discussion.

The feeding experiments conclusively indicate that *Nitzschia* and the oil obtained from it are good sources of vitamin A. The extracted oil gives a positive reaction with $SbCl_3$ and the apparent discrepancy observed by Drummond and Watson does not exist.

The extreme richness of the oil in the natural plant pigments will be clear from a study of Table II. The greater part of the colour is due to chlorophyll and xanthophyll, both of which are inactive as a source of vitamin A [Willimott and Moore, 1927]. Carotene, whose relation to vitamin A is supported by increasing evidence, is also present, and it is possible that the greater part of the physiological activity of the oil is due to this pigment. A spectroscopic examination would have indicated the part of the activity due to carotene and the part due to vitamin A, but unfortunately enough material was not available for this to be made. If we compare the biological activities of this pigmented oil and carotene (rat dose 5 mg. and 0·005 mg. respectively) and their respective yellow colours (10,000 and 300,000 Lovibond units in 10 % dilution in 1 cm. cell), we find that the whole of the biological activity of the oil can be accounted for if 3 % of the yellow pigment present be carotene. This is interesting when considered with the earlier observations of Steenbock and his colleagues pointing to the association of the lipochrome pigments and vitamin A activity of vegetable sources; of Coward [1923] that the lipochrome chiefly concerned in this association was carotene; the more recent work on the activity of carotene; and our own observations and that of Moore [1929, 1930] that from the livers of rats fed on large quantities of carotene (0·02–0·05 mg. per day) a colourless or a lightly coloured fat is obtained which gives an intense colour reaction with $SbCl_3$. It will probably not be far from the truth if it is suggested that these diatoms are the ultimate source of vitamin A of the fish-liver oils, and that a similar change takes place in the cod or the intermediate fauna giving rise to the stores of the colourless vitamin A present in the liver oils. It also explains why liver oils of fish may be very pale in colour and at the same time very rich in vitamin A, a fact which had rather perplexed the earlier supporters of the view that vitamin A was related to a lipochrome pigment.

Summary.

1. The oil obtained from a marine diatom (*Nitzschia closterium*) growing in pure culture, has been studied both colorimetrically and biologically as a source of vitamin A.

2. It has been found that the oil is very potent in this respect and gives a strong colour reaction with $SbCl_3$.

3. The greater part of the biological activity of the oil is probably due to carotene, synthesised by the organism, and extracted with the oil.

In conclusion, I desire to thank Prof. J. C. Drummond for his kind help and advice throughout the course of this investigation, and the Medical Research Council for a grant in aid of the expenses.

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XCVI. THE COMPOSITION OF THE FATTY ACIDS PRESENT AS GLYCERIDES IN THE LIVER OIL OF THE THRESHER SHARK (*ALOPOECIA VULPES*).

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(*Received May 27th, 1930.*)

In a recent communication [Guha, Hilditch and Lovern, 1930] an account was given of quantitative analyses of the fatty acids of a series of fish-liver oils. When these were subsequently arranged according to their biological families, the oils of the elasmobranch group, including shark, dogfish and skate, lent support to the view that there was a definite and close relation between the amount of squalene present in the non-glyceride portion of the oil and the nature of the fatty acid constituents. When the amount of squalene was high, as in *Scymnorhinus lichia*, the acids were almost exclusively monoethylenic, and contained, in contrast to other fish-liver oils examined, such as those of the Teleostomi group, appreciable amounts of an unsaturated acid containing twenty-four carbon atoms (selacholeic acid). As the percentage of squalene present decreased there was a steady increase in the degree of unsaturation of the fatty acids, together with a steady diminution in the amount of C_{24} acid present, *e.g. Squalus acanthias*, with *ca.* 4 % of squalene (10·5 % unsaponifiable). In the liver oil of the skate (*Raia maculata*) studied, squalene was practically entirely absent, the unsaturation of the C_{20} and C_{22} groups of acids was exceedingly high, and acids of the C_{24} group were not present. It is highly desirable that further information regarding the liver oils of members of this group should be obtained, since the correlation outlined above rests at present on but a few instances.

In this connection the author was fortunate to obtain access to the fatty acid portion of the liver oil of a thresher shark (*Alopoecia vulpes*), the liver of which had been secured by Prof. J. C. Drummond of University College, London, to whom, with Mr L. C. Baker, the writer desires to express his thanks for the information given regarding the crude oil. This particular shark was caught off the Dorset coast on the 18th of June, 1928, and landed at Abbotsbury. The weight of the fish was 4 cwt., and the liver, procured on the 20th of June, weighed 4 kg. The final yield of oil (prepared by steaming) was 650 g. This was of a golden brown colour, with some separation of crystalline matter on standing. The characteristics of the oil were as follows:

% free fatty acids (as oleic)	0·20
Saponification value	181
Iodine value (Dam)	176
Refractive index 20°	1·4741
% unsaponifiable	1·83

The iodine value of the unsaponifiable matter was 102 and it contained 21·8 % of cholesterol; this corresponds with an iodine value for the rest of the unsaponifiable matter of about 112, as against 370 for squalene, so that the amount of squalene (if any) in thresher shark-liver oil must be very low. From this standpoint it is therefore in the same category as skate-liver oil (*Raia maculata*), and, as the following analysis shows, the fatty acid composition is also remarkably similar to that of skate-liver oil. The method of analysis was precisely the same as that given for the other oils [Guha *et al.*, 1930] and only the actual data are given here. It may be noted in passing that in both skate- and thresher shark-liver oils there was very little tendency to polymerisation, although both oils had been kept for over a year before analysis, and both contained a large proportion of acids of a very high degree of unsaturation.

Experimental.

Fatty acids; I.V. 181·4, sap. eq. 289·6.

Lead salt separation.

	g.	%	I.V. (of corresponding methyl esters)
"Solid" acids S	51·0	24·5	38·64
"Liquid" acids L	149·0	74·5	236·3

Fractionation of methyl esters.

Primary fractions					Refractionations				
		B.P./1 mm.					B.P./1 mm.		
No.	g.	°C.	S.E.	I.V.	No.	g.	°C.	S.E.	I.V.
(i) *Esters of "solid" acids S* (49·5 g.).									
S 1	35·37	100–130	261·0	10·69					
S 2	5·73	130–145	289·6	73·99					
S 3	8·00	Residue	325·8	143·2					
(Fraction S 1 (16·72 g.) gave 15·00 g. of saturated esters (S.E. 259·4) on oxidation.)									
(ii) *Esters of "liquid" acids L* (145 g.).									
L 1	39·79	127–140	272·1	113·6	L 11	8·49	105–122	249·7	68·0
					L 12	18·92	122–128	270·4	104·4
					L 13	8·07	Residue	292·1	188·8
L 2	34·36	140–158	306·5	218·6	L 21	4·42	120–139	290·6	148·5
					L 22	19·09	139–143	304·5	204·4
					L 23	6·27	Residue	319·6	286·9
L 3	56·44	158–175	329·6	320·9	L 31	4·20	132–165	307·3	276·4
					L 32	29·54	165–170	327·3	320·3
					L 33	10·15	170–175	331·8	342·2
					L 34	5·66	Residue	331·1	326·5
L 4	13·83	Residue	346·3*	290·2					

* Saponification equivalent of residual esters, freed from unsaponifiable matter, L 4 : 337·7.

Estimated composition of fatty acids.

	"Solid" acids S (24·5 %)	"Liquid" acids L (74·5 %)	Total %	Fatty acids %	Mean unsaturation
Saturated					
Myristic	5·8	1·6	7·4	7·4	
Palmitic	11·3	—	11·3	11·3	
Stearic	0·2	—	0·2	0·2	
Unsaturated					
C_{14} group	—	1·6	1·6	1·6	
C_{16} "	—	12·0	12·0	12·0	(− 2·0 H)
C_{18} "	4·0	15·1	19·1	19·2	(− 3·4 H)
C_{20} "	3·7	27·2	30·9	31·0	(− 6·6 H)
C_{22} "	0·5	16·8	17·3	17·3	(− 10·5 H)
Unsaponifiable	—	0·2	0·2	—	

It will be observed that the results for thresher shark-liver oil are in entire agreement with the theory already tentatively advanced, but it should be reiterated that the number of cases examined up to the present is insufficient for this generalisation to be regarded as proved. More data are evidently very desirable before any deductions are seriously advanced, but some reference to the many problems suggested by such a relation (if proved) may perhaps be permitted. For instance, is the relation between the squalene content and the fatty acids definite for any particular species? Only one sample of oil from each species has been fully examined, whereas it is known that the unsaponifiable content of particular members of the Elasmobranchi may vary between wide limits. Thus, unsaponifiable matter between the limits of 4 and 32·9 % has been found in the liver oil of the dogfish (*Squalus acanthias*), a mixture of squalene and cholesterol being present in many cases [Grün and Halden, 1929]; liver oils of the basking shark (*Cetorhinus maxima*) have been observed [Grün and Halden, 1929] to contain from 20 % to as much as 56 % of unsaponifiable matter (of which approximately one-half is frequently squalene); whilst, similarly, in oils from livers of *Scymnorhinus lichia* [Channon, 1928] the unsaponifiable content varied from 48·5 % to 81·5 %.

There is thus no reason to assume that skate-liver oil, for instance, will always be found to contain negligible amounts of squalene. Channon [1928] has given evidence which suggests that the squalene present in these fish-liver oils is not taken in the food, but is synthesised by the fish. To mention two results: (1) there is no squalene present in the various samples of plankton examined; (2) the feeding habits of all members of the Squalidae family appear to be similar, and yet squalene is present in large amount in some, and entirely absent from others.

One has also to take into account, if accepting the theory of synthetic origin, the fact that squalene is by no means the only unsaponifiable matter present in elasmobranch oils. Other substances frequently found (in varying amounts, sometimes alone and sometimes with squalene) include hydrocarbons (*e.g. iso*octadecane, decane, etc.), cholesterol, and ethers such as batyl, chimyl and selachyl alcohols. It has yet to be determined whether there

is any relation between the presence or absence of these substances and the composition of the accompanying fatty acids.

In conclusion, the author wishes to express his thanks and appreciation to Prof. T. P. Hilditch for his kind interest and advice throughout the course of this work.

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XCVII. VITAMIN A OF BUTTER.

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(*Received May 10th, 1930.*)

It is now clear that the physiological action of the vitamin A of liver fats can be reproduced, or at any rate closely simulated, by the plant pigment carotene [Euler, Euler and Hellström, 1928; Euler, Euler and Karrer, 1929; Moore, 1929, 1, 2; 1930; Collison, Hume, Smedley-MacLean and Smith, 1929], and it therefore becomes important to ascertain

(*a*) whether butter contains either or both of these substances;

(*b*) the relative importance of these substances, if both are present;

(*c*) whether the quantities of either or both vary with seasonal, dietary or other conditions.

In the present paper an account is given of a preliminary survey of this question wherein we have applied spectrographic methods as a means of arriving at a decision concerning the above points.

Before dealing with the absorption spectra of the butter extracts themselves it is necessary to recall that both carotene and the vitamin A of liver fats can be recognised in extracts of non-saponifiable matter by their absorption spectra and also by the spectral location of the principal absorption band in the blue solutions obtained by the well-known antimony trichloride reaction.

Absorption spectra.

A solution of carotene in chloroform shows maxima at 492, 462, 436, 348 and 279 $\mu\mu$, whilst in alcohol the visible bands appear at 480, 451 and 427 $\mu\mu$. Whereas the ultra-violet absorption of carotene is weak relatively to the visible absorption [Dulière, Morton and Drummond, 1929], liver oils and non-saponifiable extracts on the other hand show negligible absorption in the visible, but in the ultra-violet a strong band with a maximum near 328 $\mu\mu$, the intensity of absorption at the head of the band affording a trustworthy measure of the vitamin A potency [Morton and Heilbron, 1928, 1930].

The non-saponifiable matter from a sample of New Zealand butter exhibited clear maxima at 492, 462 and 432 $\mu\mu$ when the solvent was chloroform and at 480, 452 and 427 $\mu\mu$ when alcohol was used. Comparison of the intensity of absorption for pure carotene and for the butter extracts indicated that the latter contained about 1 part in 200 of the pigment. The butter extract is, however, sharply differentiated from the absorption spectrum of carotene

itself by the fact that it exhibits rapidly increasing intensity of absorption on the ultra-violet side of 380 $\mu\mu$ (Fig. 1). The absorption curve shows a marked

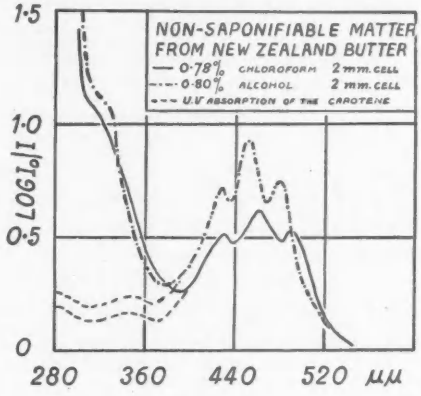

Fig. 1.

inflexion at 310–340 $\mu\mu$, and there can be little doubt that the substance responsible for this is identical with the vitamin A of liver oils. In addition to this, however, another substance which shows a clear maximum in the neighbourhood of 272–275 $\mu\mu$ (Fig. 3) is also present in the butter extract. The observed ultra-violet absorption of the butter extract is therefore a summation of three separate effects:

(1) the ultra-violet absorption of the carotene, the intensity being deducible from the visible absorption of the butter extract by utilising the known absorption curve for pure carotene;

(2) the ultra-violet absorption of the unidentified substance characterised by a maximum near 272 $\mu\mu$;

(3) the ultra-violet absorption of the distinct vitamin A which has hitherto been associated mainly with fish-liver oils.

The impossibility of distinguishing quantitatively between the two types of ultra-violet absorption restricts the interpretation of the absorption spectrum of butter extracts in the ultra-violet to fixing an upper limit to the potency of the vitamin A. Calculations indicate that the non-saponifiable matter from the New Zealand butter contains, in addition to about 0·5 % of carotene, sufficient vitamin A to make its potency not more than about six times that of a good cod-liver oil[1].

An examination of the non-saponifiable matter of Danish butter (commercial sample) provides evidence of a much smaller carotene content (Fig. 2), this being of the order of 0·1–0·15 %; as regards the vitamin A content, the upper limit does not appear to be very different from that for the New Zealand

[1] This figure is arrived at by comparing the intensities of absorption at 328 $\mu\mu$ for equal concentrations and all thicknesses of cod-liver oil and butter concentrate.

butter. On the other hand the concentration of the material responsible for the band near 272 $\mu\mu$ is very definitely greater (Fig. 3), and this last observation would tend to show that the true vitamin A content of the Danish butter is

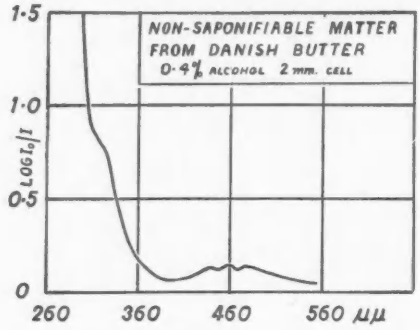

Fig. 2.

lower than that of the New Zealand butter, the high concentration of the unidentified substance being probably responsible to some extent for the intensity of absorption near 320 $\mu\mu$.

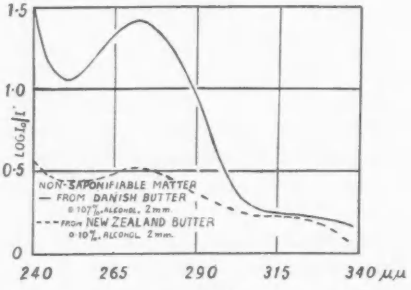

Fig. 3.

Antimony trichloride reaction.

The blue colour obtained with pure carotene is characterised by a well-defined band at 591 $\mu\mu$ [Dulière, Morton and Drummond, 1929], whilst that obtained with non-saponifiable matter from liver oils shows a sharp maximum at 624 $\mu\mu$ [Morton and Heilbron, 1928, 1930]. Using the New Zealand extract both these maxima are shown clearly at 624 and 592 $\mu\mu$. The intensity of the 624 $\mu\mu$ band was about five times that associated with an equal weight of a good cod-liver oil, whilst the less intense band at 592 $\mu\mu$ indicated a maximal carotene content of 2 %. This figure is undoubtedly too high, as the liver oil concentrates (in which carotene is certainly not present in any significant amounts) often give high, if somewhat unselective, absorption at 592 $\mu\mu$.

The non-saponifiable matter from Danish butter gave a good colour with antimony trichloride, the tint being definitely more green than blue. A very sharp band at 620–622 $\mu\mu$ was seen but no band at 592 $\mu\mu$ could be recognised. This was not surprising as the spectrographic data had already proved that the carotene content was relatively low. The intensity of the 620–622 $\mu\mu$ band indicated that the potency of the Danish butter extract was about 2·45 times that of cod-liver oil.

It will thus be seen that the direct absorption spectra data and the study of the antimony trichloride blue colours give a consistent account of the properties of the non-saponifiable matter from butter.

The recognition of carotene together with the distinct vitamin A of liver fats in a product derived from milk is of importance and lends support to Moore's view [1930] that carotene can be converted *in vivo* to vitamin A.

It should be emphasised that the butters used were not chosen as representative of their respective classes, and many more samples will need to be studied before any generalisations can be made.

We are indebted to Messrs Joseph Nathan and Co., Ltd. for the preparation of the butter extracts and to the Department for Scientific and Industrial Research for a grant which has enabled this investigation to be carried out.

Summary.

The non-saponifiable matter from butter has been shown spectroscopically to contain carotene, vitamin A, and an unidentified substance characterised by selective absorption at 272 $\mu\mu$. The carotene and vitamin A potency can be estimated with some degree of accuracy.

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XCVIII. THE ACTION OF THE CINCHONA AND CERTAIN OTHER ALKALOIDS IN BIRD MALARIA.

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(*Received May 27th, 1930.*)

Although cinchona bark first appeared in Europe about 1639 and the first cinchona alkaloid, quinine, was isolated from it in 1820 and has been manufactured and used in medicine ever since, there are still many unsolved problems in connection with the use of cinchona alkaloids in malaria.

Some of these questions are of great practical importance. Thus it has been assumed that for the treatment of malaria the best of the four principal cinchona alkaloids is quinine, and over 90 % of the bark now produced from cinchona plantations is bark cultivated to give high yields of quinine.

In the last few years misgivings have arisen as to whether this is the best policy to adopt, and it has even been suggested that it might be advisable to go back to the plan adopted in British India and Ceylon in the early days of cinchona planting and to cultivate the hardy *Cinchona succirubra* species, which yields a mixture of alkaloids, in which quinine and cinchonidine predominate, and to prepare this mixture as "quinetum" for the large scale treatment of malaria [Gage, 1925]. As a basis for the serious discussion of such questions it is desirable that the relative therapeutic values of the principal crystalline alkaloids found in cinchona bark, viz. quinine, quinidine, cinchonidine and cinchonine, should be determined. Hitherto, the only method of making such a comparison has been by clinical trials. The difficulties of making such comparative trials are explained in a report by the Medical Research Council [1925] dealing with the relative values of quinine and quinidine. Probably the difficulties experienced in the case of these two bases have so far precluded extension of the investigation to a similar comparison between quinine and the other readily procurable cinchona alkaloids, cinchonidine, cinchonine, and the reduction product of quinine, hydroquinine. If, in such a comparative trial, cinchonidine proved to be as useful as quinine, there would be a *prima facie* case for the use of "quinetum" in place of quinine.

The relative values of the four alkaloids are also of importance in connection with the use of "cinchona febrifuge," which consists of the total

residual alkaloids of cinchona bark, left after the removal of as much of the crystallisable alkaloids as manufacturers normally require to meet commercial demands. Large quantities of this material are now used in malarial countries and it is, as might be expected, of varying quality [Howard, 1925]. Its use has numerous advocates, but before this can be placed on a scientific basis, a satisfactory method of analysis for cinchona febrifuge is required so that definite standards can be set up for it [Goodson and Henry, 1930]. These standards ought to take into account the relative therapeutic values of the components as well as industrial convenience.

It is also sometimes suggested that the special value attributed by some authorities to the antimalarial activity of cinchona febrifuge is due to the presence of some highly active and so far undiscovered component [Prain, 1925].

Apart from these practical problems, there are also questions of purely scientific interest. Much discussion has taken place as to the particular part of the complex structure of the quinine molecule which determines its antimalarial activity, and certain tentative conclusions have been reached based partly on *in vitro* tests with protozoa and partly on limited clinical trials, but there is still no certainty about these conclusions, though they may have an important bearing on the synthesis of new antimalarial drugs [Kaufmann, 1926; Giemsa, Weise and Tropp, 1926; Shaw, 1928].

Further, in the course of the last 10 years many "modified cinchona alkaloids" have been prepared in the laboratory and for some of these claims have been made for increased action in malaria.

It seemed to us that the technique standardised by Roehl [1926] for testing the efficacy of drugs in bird malaria might be used with advantage to investigate some of these questions since it was by this means that the efficacy of "plasmoquin" in malaria was discovered, though the case of "dimeplasmin" [Green, 1929], which was investigated in the first instance by the same method, indicates that positive results in bird malaria may not always indicate that a drug will be efficacious in human malaria.

Among the "modified cinchona alkaloids" the series of "quitenines" obtained by the gentle oxidation of the four principal cinchona alkaloids, whereby the CH : CH_2 group in the quinuclidine nucleus (*R'* in the generalised formula, p. 876) is oxidised to a carboxyl group, occurred to us as a promising basis for a homologous series of products, the "alkylquitenines," in which the value of Roehl's technique for dealing with questions of this kind could be tested. It is, for example, well known that in *in vitro* tests of bactericidal agents action increases as a homologous series is ascended up to a maximum and then diminishes. In the series of 4-*n*-alkylresorcinols, maximal effect is reached at hexylresorcinol [Johnson and Lane, 1921; Dohme, Cox and Miller, 1926], in the *p*-*n*-alkylphenols at *n*-amylphenol [Coulthard, Marshall and Pyman, 1930] and in the alkyldihydrocupreines at octyldihydrocupreine [Schäffer, 1917; Bieling, 1918]. But this principle, though it is traceable in the results

of a number of investigations [*e.g.* Morgenroth and Tugendreich, 1916], has not been established with the same certainty in *in vivo* therapeutic tests. It has, however, already been shown that in a short series of cupreine ethers the following changes in activity in bird malaria occur. The figures in brackets give the retardation of attack in days [Giemsa, Weise and Tropp, 1926]:

Cupreine (0–4).

Cupreine ethers:

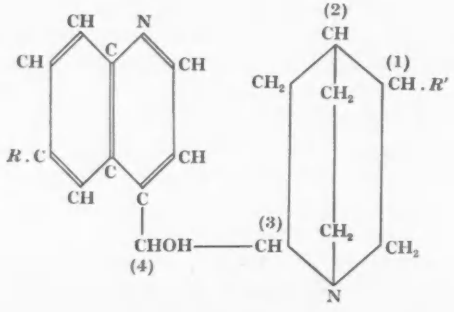
Methyl: quinine (12–13); quinidine (12).
Ethyl: quinethyline (8–40).
Propyl: quinpropyline (4–10).
Amyl: quinamyline (7–11).

With regard to quitenine itself it has been clearly established that this substance is inactive in both human and bird malaria, but that activity is recovered in the ethyl ester [Giemsa, 1926]; investigation in this series does not seem to have been carried beyond this single observation.

Finally, there is a considerable number of natural drugs other than cinchona bark and its constituent alkaloids which have local reputations as remedies for malaria or as febrifuges. Some of these, such as the *Alstonia* barks, have been recognised in Pharmacopoeias, but the evidence for their therapeutic value is slender. The discovery of a highly active substance among them might provide a new starting-point for the synthesis of antimalarial drugs, and it seemed reasonable to suppose that Roehl's method might at least enable any promising product to be picked out of this group for further examination. The present paper records the results of attempts to apply this method to the solution of some of these problems.

General formula for cinchona alkaloids.

As it will frequently be necessary to refer to derivatives of the various cinchona alkaloids, it may be convenient at this stage to give the following generalised formula for the principal cinchona alkaloids:



R = H in cinchonine, cinchotenine, dihydrocinchonine, cinchonidine, cinchotenidine and dihydrocinchonidine: in all other cases it is $\cdot OCH_3$, except in cupreine where it is $\cdot OH$ and the cupreine alkyl ethers, quinethyline ($\cdot OC_2H_5$); quinpropyline ($\cdot OC_3H_7$); quinamyline ($\cdot OC_5H_{11}$).

R' = $\cdot CH:CH_2$ in cinchonine, cinchonidine, quinine and quinidine

= $\cdot CH_2.CH_3$ in the dihydro-reduction products of these four alkaloids

= COOAlk. for the alkyl-quitenines, quitenidines, cinchotenines and cinchotenidines; Alk. = methyl, ethyl, propyl, etc.

Carbon atoms 1, 2, 3, 4 are all dextrorotatory in cinchonine and its derivatives and in quinidine and its derivatives: carbon atoms 1 and 2 are dextrorotatory and 3 and 4 laevorotatory in cinchonidine and quinine and their derivatives [King, 1922].

Preparation of the drugs.

The cinchona alkaloids used were laboratory stock, which had been used for other investigations and were of satisfactory purity. The hydrogenated alkaloids were prepared from them by reduction with hydrogen in presence of palladium as a catalyst. The esters of quitenine and of the analogous acids derived from cinchonidine, quinidine and cinchonine were prepared by the general method described below. Many of them are new and consequently it is desirable to include a brief record of their chemical characteristics; this is given below, the names of the new substances being printed in italics.

The *iso*quinoline alkaloids referred to on p. 888 were laboratory specimens prepared mainly by Dr F. L. Pyman, F.R.S., in the course of former investigations carried out in one of these laboratories.

The other preparations referred to on pp. 887, 888 are mainly derived from chemical investigations now in progress. The various "total alkaloids as hydrochlorides" were prepared by extracting the total alkaloids of the plant specified and neutralising these with hydrochloric acid to form a neutral solution of known strength. This rule of using all the drugs as neutral hydrochlorides was only diverged from when the neutral hydrochloride was too sparingly soluble to enable a dose completely in solution in 0·5 cc. to be given, and in such cases either the acid hydrochloride or a still more soluble salt, such as the acetate or the ethylsulphonate, was used.

Esters of quitenine, quitenidine, cinchotenine, cinchotenidine.

The esterifications were effected with good yields, in the manner described for methylquitenine, unless otherwise stated.

Methylquitenine. Dry hydrogen chloride was passed into a mixture of anhydrous quitenine (10 g.) and methyl alcohol (50 cc.) heated on the water-bath for 2 hours. After removal of methyl alcohol by distillation, the aqueous solution of the residue was made alkaline with sodium carbonate solution, and

the methyl ester extracted by chloroform, the small amount of unchanged quitenine, which separated, being removed by filtration.

The base was not obtained crystalline. It softened at 165° (corr.), melted at 170° (corr.) and had $[\alpha]_D^{20°} - 130°$ ($c = 2{\cdot}24$ in chloroform). (Found: C, 67·1; H, 6·9. $C_{20}H_{28}O_4N_2$ requires C, 67·4; H, 6·8 %.) *Methylquitenine monohydrochloride* was prepared by neutralising a solution of the base in alcohol with dilute hydrochloric acid. It crystallised in plates (frothing at 85°) with difficulty from methyl alcohol on adding dry ether, and once crystallised it could readily be recrystallised from benzene in needles, frothing at 96°. The anhydrous salt commenced to change at 153° (corr.) and gradually frothed. It had $[\alpha]_D^{21°} - 189°$ ($c = 0{\cdot}994$ in water) and was very soluble in water. (Found in air-dried crystals from methyl alcohol-ether: loss at 105°[1] in a vacuum, 7·4. $C_{20}H_{24}O_4N_2$, HCl, $2H_2O$ requires H_2O, 8·4 %. Found in dry salt: C, 60·9; H, 6·8; Cl, 8·8; MeO, 15·6. $C_{20}H_{24}O_4N_2$, HCl requires C, 61·1; H, 6·4; Cl, 9·0; 2MeO, 15·8 %.)

Ethylquitenine crystallises from wet ether or alcohol, or better from a mixture of chloroform and ethyl acetate, in anhydrous needles, M.P. 201° (corr.), and has $[\alpha]_D^{20°} - 141°$ ($c = 1{\cdot}5935$ in chloroform). (Found: C, 67·9; H, 7·6. $C_{21}H_{26}O_4N_2$ requires C, 68·1; H, 7·1 %.) Ethylquitenine monohydrochloride was recrystallised from acetone. The dry salt sintered at 158° (corr.), melted at 162° (corr.) and had $[\alpha]_D^{16°} - 206°$ ($c = 0{\cdot}988$ in water). It is very soluble in water. (Found: Cl, 8·6. $C_{21}H_{26}O_4N_2$, HCl requires Cl, 8·7 %.) Ethylquitenine dihydrochloride crystallises from alcohol. It is hygroscopic and very soluble in water. (Found in dry salt: Cl, 15·7. $C_{21}H_{26}O_4N_2$, 2HCl requires Cl, 16·0 %.)

n-*Propylquitenine* separated from acetone in anhydrous crystals, apparently tetrahedra, melting at 170° (corr.) and having $[\alpha]_D^{17°} - 143°$ ($c = 2{\cdot}048$ in chloroform). (Found: C, 68·8; H, 7·6. $C_{22}H_{28}O_4N_2$ requires C, 68·7; H, 7·3 %.) n-*Propylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 89°. (Found in air-dried salt: loss at 100° in a vacuum, 8·4. $C_{22}H_{28}O_4N_2$, HCl, $2H_2O$ requires H_2O, 7·9 %.) The anhydrous salt sintered at 125° (corr.), melted at 187° (corr.) and had $[\alpha]_D^{20°} - 225°$ ($c = 1{\cdot}01$ in water). Solubility 1 in 7 of water at 23°. (Found: Cl, 8·3. $C_{22}H_{28}O_4N_2$, HCl requires Cl, 8·4 %.)

iso*Propylquitenine* crystallised from benzene in anhydrous clusters of plates, sintering at 184° (corr.), melting at 189° (corr.) and having $[\alpha]_D^{22°} - 142°$ ($c = 2{\cdot}011$ in chloroform). (Found: C, 68·8; H, 7·6. $C_{22}H_{28}O_4N_2$ requires C, 68·7; H, 7·3 %.) iso*Propylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 88° (corr.). (Found in air-dried salt: loss at 105°

[1] When the air-dried substances described in this paper underwent change below 105°, they were dried first at a low temperature and finally at the temperature recorded. In a number of cases the salts crystallised with benzene, which was partly replaced by water on exposure to air, the products becoming sticky. In these cases the loss on drying does not correspond with any definite number of molecules of either solvent.

in a vacuum, 11·5 %.) The anhydrous salt sintered at 126° and gradually melted. It had $[\alpha]_D^{20°} - 231°$ ($c = 1·008$ in water). Solubility in water 1 in 19 at 20°. (Found: Cl, 8·4. $C_{22}H_{28}O_4N_2$, HCl requires Cl, 8·4 %.)

n-*Butylquitenine* crystallised from a mixture of benzene and light petroleum (B.P. 60–80°) in anhydrous silky needles, sintering at 137° (corr.), melting at 142° (corr.) and having $[\alpha]_D^{22°} - 143°$ ($c = 2·00$ in chloroform). (Found: C, 68·9; H, 7·8. $C_{23}H_{30}O_4N_2$ requires C, 69·3; H, 7·6 %.) n-*Butylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 88°. (Found in air-dried salt: loss at 105° in a vacuum, 11·0 %.) The anhydrous salt sintered at 110° and gradually melted. It had $[\alpha]_D^{21°} - 227°$ ($c = 0·631$ in water) and solubility 1 in 123 of water at 20°. (Found: Cl, 8·2. $C_{23}H_{30}O_4N_2$, HCl requires Cl, 8·2 %.) n-*Butylquitenine dihydrochloride* was not obtained crystalline. The dried substance, which softened at 168° (corr.) and frothed at 178° (corr.), was hygroscopic and had $[\alpha]_D^{18°} - 250°$ ($c = 2·045$ in water). (Found: Cl, 14·8. $C_{23}H_{30}O_4N_2$, 2HCl requires Cl, 15·0 %.)

iso*Butylquitenine* crystallised from benzene in matted needles, softening at 147° (corr.), melting at 154° (corr.) and having $[\alpha]_D^{20°} - 144°$ ($c = 2·02$ in chloroform). (Found: C, 68·9; H, 7·8. $C_{23}H_{30}O_4N_2$ requires C, 69·3; H, 7·6 %.) iso*Butylquitenine monohydrochloride* crystallised from benzene in needles, frothing at 93° (corr.). (Found in air-dried salt: loss at 100° in a vacuum, 9·2. $C_{23}H_{30}O_4N_2$, HCl, $2H_2O$ requires H_2O, 7·7 %.) The anhydrous salt sintered at 125°, melted at 248° (corr.) and had $[\alpha]_D^{20°} - 223°$ ($c = 1·52$ in water). Solubility 1 in 66 of water at 20°. (Found: Cl, 8·1. $C_{23}H_{30}O_4N_2$, HCl requires Cl, 8·2 %.)

n-*Amylquitenine* crystallised from a mixture of acetone and ligroin in needles, softening at 129° (corr.), melting at 135° (corr.) and having $[\alpha]_D^{22°} - 135°$ ($c = 2·00$ in chloroform). (Found: C, 70·0; H, 8·1. $C_{24}H_{32}O_4N_2$ requires C, 69·9; H, 7·8 %.) n-*Amylquitenine monohydrochloride* crystallised from benzene in minute needles, softening at about 40° and melting at 194° (corr.). (Found in air-dried salt: loss at 100° in a vacuum, 7·6 %. $C_{24}H_{32}O_4N_2$, HCl, $2H_2O$ requires H_2O, 7·4 %.) The anhydrous salt softened at about 115°, resolidified and melted at 195° (corr.). It had $[\alpha]_D^{20°} - 217°$ ($c = 0·92$ in water) and solubility 1 in 107 of water at 20°. (Found: Cl, 7·9. $C_{24}H_{32}O_4N_2$, HCl requires Cl, 7·9 %.)

iso*Amylquitenine* crystallised from benzene in matted needles, softening at 154° (corr.), melting at 160° (corr.) and having $[\alpha]_D^{21°} - 136°$ ($c = 1·989$ in chloroform). (Found: C, 69·0; H, 8·0. $C_{24}H_{32}O_4N_2$ requires C, 69·9; H, 7·8 %.) iso*Amylquitenine monohydrochloride* crystallised well from benzene. The air-dried substance frothed at 90°. (Found in air-dried salt: loss at 105° in a vacuum, 19·8 %.) The anhydrous salt softened at 122° (corr.) and gradually decomposed. It had $[\alpha]_D^{17°} - 222°$ ($c = 0·804$ in water) and solubility 1 in 112 of water at 18°. (Found: Cl, 7·9. $C_{24}H_{32}O_4N_2$, HCl requires Cl, 7·9 %.)

Octylquitenine was obtained in about 10 % yield only, although the proportion of octyl alcohol (methyl-*n*-hexylcarbinol) to quitenine was increased

to 50 cc. of alcohol to 5 g. of quitenine, and the esterification was carried out at 110° for 6 hours. The base was not obtained crystalline. *Octylquitenine monohydrochloride* crystallised from benzene in needles, softening at 166° (corr.) and melting at 181° (corr.). (Found in air-dried salt: loss at 105° in a vacuum, 3·6 %. $C_{27}H_{38}O_4N_2$, HCl, H_2O requires H_2O, 3·5 %.) The anhydrous salt had $[\alpha]_D^{21°} - 191°$ ($c = 0{\cdot}244$ in water) and solubility of 1 in 410 of water at 21°. (Found: Cl, 7·2. $C_{27}H_{38}O_4N_2$, HCl requires Cl, 7·2 %.) *Octylquitenine dihydrochloride* was not obtained crystalline. It softened at 136° (corr.) and melted at 164° (corr.). A 1 % aqueous solution could be prepared. (Found: Cl, 13·2. $C_{27}H_{38}O_4N_2$, 2HCl requires Cl, 13·4 %.)

Benzylquitenine was obtained in a yield of about 20 %. After the esterification, water was added and the unchanged benzyl alcohol and benzyl chloride extracted by chloroform. The chloroform solution was washed with water and the washings added to the main acid aqueous solution of the base and unchanged quitenine. The base was liberated by sodium carbonate, extracted by chloroform and recrystallised from a mixture of benzene and light petroleum. The base usually separates as a jelly which changes into anhydrous needles, sintering at 157° (corr.) and melting at 161° (corr.). It had $[\alpha]_D^{24°} - 128°$ ($c = 2{\cdot}002$ in chloroform). (Found: C, 71·7; H, 6·9. $C_{26}H_{28}O_4N_2$ requires C, 72·2; H, 6·5 %.) *Benzylquitenine monohydrochloride* crystallised from alcohol in anhydrous prisms, melting at 233° (corr.), frothing at 238° (corr.), having $[\alpha]_D^{20°} - 175°$ ($c = 0{\cdot}4$ in water) and solubility 1 in 250 of water at 20°. (Found: C, 66·1; H, 6·3; Cl, 7·4. $C_{26}H_{28}O_4N_2$, HCl requires C, 66·6; H, 6·2; Cl, 7·6 %.) *Benzylquitenine dihydrochloride* was not obtained crystalline. It softened at about 160° and melted with frothing at about 200° (corr.). A 10 % aqueous solution could be prepared. (Found: Cl, 13·9. $C_{26}H_{28}O_4N_2$, 2HCl requires Cl, 14·0 %.)

Ethylquitenidine crystallised from acetone in six-sided prisms, sintering at 98° and frothing at 107° (corr.). (Found in air-dried crystals: loss at 120° in a vacuum, 7·4 %. $C_{21}H_{26}O_4N_2$, $2H_2O$ requires H_2O, 8·9 %.) The anhydrous base softened at 131° (corr.), melted at 139° (corr.) and had $[\alpha]_D^{20°} + 163°$ ($c = 2{\cdot}143$ in chloroform). (Found: C, 67·9; H, 7·2. $C_{21}H_{26}O_4N_2$ requires C, 68·1; H, 7·1 %.) *Ethylquitenidine monohydrochloride* crystallised from acetone in anhydrous, apparently cubical, crystals, melting at 242° (corr.), having $[\alpha]_D^{17°} + 151°$ ($c = 1{\cdot}0296$ in water) and solubility 1 in 23 of water at 23°. (Found: Cl, 8·7. $C_{21}H_{26}O_4N_2$, HCl requires Cl, 8·7 %.)

iso*Amylquitenidine monohydrochloride* crystallised from benzene. (Found in air-dried salt: loss at 105° in a vacuum, 2·1 %.) The anhydrous salt melted at 245° (corr.), had $[\alpha]_D^{22°} + 132°$ ($c = 0{\cdot}958$ in water) and solubility in water of 1 in 104 at 22°. (Found: Cl, 7·9. $C_{24}H_{32}O_4N_2$, HCl requires Cl, 7·9 %.)

Ethylcinchotenidine was obtained in 50 % yield under the usual conditions of esterification. It crystallised from acetone in anhydrous rhombic plates, softening at 177° (corr.), melting at 180° (corr.) and having $[\alpha]_D^{20°} - 115°$ ($c = 2{\cdot}081$ in chloroform). (Found: C, 70·3; H, 7·2. $C_{20}H_{24}O_3N_2$ requires

C, 70·5; H, 7·1 %.) *Ethylcinchotenidine monohydrochloride* was not obtained crystalline. It softened at 120°, frothed at 180° (corr.) and had $[\alpha]_D^{20°} - 165°$ ($c = 1{\cdot}3408$ in water). It is very soluble in water. (Found: Cl, 9·6. $C_{20}H_{24}O_3N_2$, HCl requires Cl, 9·4 %.)

iso*Amylcinchotenidine monohydrochloride* was not obtained crystalline. It melted at about 152°, had $[\alpha]_D^{21°} - 151°$ ($c = 3{\cdot}788$ in water) and solubility 1 in 26 of water at 21°. (Found: Cl, 8·5. $C_{23}H_{30}O_3N_2$, HCl requires Cl, 8·5 %.)

Ethylcinchotenine crystallised from chloroform in anhydrous needles, melting at 213–214° (corr.) and having $[\alpha]_D^{23°} + 151°$ ($c = 2{\cdot}012$ in chloroform). (Found: C, 70·2; H, 7·5. $C_{20}H_{24}O_3N_2$ requires C, 70·5; H, 7·1 %.) *Ethylcinchotenine monohydrochloride* crystallised from alcohol in sheaves of plates, frothing at 245° (corr.). (Found in air-dried salt: loss at 105° in a vacuum, 4·6 %. $C_{20}H_{24}O_3N_2$, HCl, H_2O requires H_2O, 4·6 %.) The anhydrous salt frothed at 251° (corr.), had $[\alpha]_D^{17°} + 130°$ ($c = 0{\cdot}982$ in water) and solubility 1 in 16 of water at 17°. (Found: C, 63·7; H, 6·8; N, 7·6; Cl, 9·4. $C_{20}H_{24}O_3N_2$, HCl requires C, 63·7; H, 6·7; N, 7·4; Cl, 9·4 %.)

iso*Amylcinchotenine* crystallised from chloroform or benzene in anhydrous needles, melting at 147–149° (corr.). iso*Amylcinchotenine monohydrochloride* was not obtained crystalline. It softened at 129° (corr.), melted at 145° (corr.), had $[\alpha]_D^{20°} + 117°$ ($c = 1{\cdot}3224$ in water) and solubility 1 in 10 of water at 20°. (Found: Cl, 8·6. $C_{23}H_{30}O_3N_2$, HCl requires Cl, 8·5 %.)

The solubilities of anhydrous quitenine, quitenidine, cinchotenidine and cinchotenine in water and their optical rotation in 5 % solution in N H_2SO_4 were determined.

	Solubility	Specific rotation
Quitenine	1 in 8300 at 18°	$[\alpha]_D^{25°} - 298°$
Quitenidine	1 in 200 at 23°	$[\alpha]_D^{23°} + 258°$
Cinchotenidine	1 in 277 at 21°	$[\alpha]_D^{25°} - 207°$
Cinchotenine	1 in 233 at 19°	$[\alpha]_D^{26°} + 209°$

The 5-nitrodihydroquinine and 5-aminodihydroquinine were prepared by the methods of Jacobs and Heidelberger [1920].

Biological tests.

The biological tests[1] were made in a manner similar to that standardised by Roehl. Canaries were infected with malaria by intraperitoneal inoculation and were subsequently treated daily for 6 days with the compound to be tested. The strain of malaria used was kindly sent to us by Roehl from Elberfeld.

[1] The biological tests were made at the London School of Hygiene and Tropical Medicine, where one of us (M.) was engaged in investigations on bird malaria on behalf of the Medical Research Council's Chemotherapy Committee, with the aid of grants from the Milner Research Fund and from the Medical Research Council. Our sincere thanks are due to the Director, Sir Andrew Balfour, and to Dr J. G. Thomson for the hospitality of their laboratory.

Thanks to the courtesy of Dr J. G. Thomson we have been able to examine named specimens (sent from Baltimore by Prof. R. Hegner) of the three species of bird-malaria parasite separated by Hartman [1927] and to compare them with the strain obtained from Roehl. The latter corresponded to the type named by Hartman *Plasmodium inconstans*. The doses of the compounds to be tested, dissolved in 0·5 cc. water, were administered to the canaries by means of an oesophageal tube passed into the stomach, and were proportional to the weight of the bird, a bird of 20 g. weight being taken as the standard. The first dose was given 4 hours after the malaria inoculation. As a rule only one bird was treated with each dose of the compound to be tested, and the first tests were made with the largest dose tolerated for 6 consecutive days. Evidence of malaria infection was sought by the examination of stained blood films daily or on alternate days.

In each experiment several birds, usually 20 %, were not treated and were regarded as controls. In the blood of such controls malaria parasites appeared for the first time usually on the 7th or 8th day of the experiment. The virulence of the parasite was somewhat variable, but during 2 years no seasonal variation and no progressive change in virulence was observed. In the birds used as controls during this time the incubation period varied from 3 to 11 days, and averaged 7·3 days; in 49 % the parasites appeared first on the 7th or 8th day, and in 83 % between the 6th and 9th days. Making allowance for this degree of variability in the incubation period, we have adopted an arbitrary standard for estimating the antimalarial action of the compounds tested. A delay in the appearance of parasites as compared with the controls of only 1 or 2 days has been considered negligible, a delay greater than this but not exceeding 7 days has been construed as indicating a "slight" action, and a delay of over 7 days a "moderate" action. The canaries were ordinarily kept under observation for 3 or 4 weeks. If at the end of this time they had shown no evidence of malaria, they were re-inoculated so as to determine whether they had actually been cured by the treatment or were the subjects of a latent infection. If the birds were actually cured, that is malaria-free, the effect of re-inoculation was to produce a normally acute infection, whereas if they were still infected, although not showing parasites in the blood, re-inoculation was without effect. This test presupposes that it is impossible to super-infect with *P. inconstans*, a fact which has been abundantly demonstrated both in the course of this work and by numerous previous investigators. It also assumes that in the course of the 20 days or so intervening between the end of treatment and the day of re-inoculation the drug being tested will have been eliminated from the bodies of the birds. It will be noted that on one or two occasions this assumption was probably incorrect because the first re-inoculation was without effect, whereas re-inoculation at a later date produced a normal acute infection.

The test as outlined above is clearly not a delicate one. It is probably sufficiently trustworthy to distinguish broadly between active and inactive compounds, but for the differentiation of compounds not having widely

dissimilar actions, or for the grading of a series of substances with actions of the same order, experiments would have to be made with very much larger numbers of birds.

Table I. *Cinchona alkaloids.*

Drug	Dose in mg. No. of daily doses given	Day on which parasites appeared in blood: Control birds	Day on which parasites appeared in blood: Treated birds	Antimalarial action
Quinine hydrochloride	10·0 × 6	6	—	Cured*
	5·0 × 6	6–8	11–13	Slight
	2·0 × 6	6	14–15	Moderate
	1·0 × 6	7–9	16–17	Moderate
	1·0 × 6	—	12	Slight
	1·0 × 6	6	5	None
	0·5 × 6	5	10	Slight
	0·5 × 6	7–8	9	None
	0·5 × 6	7–9	5	None
Hydroquinine dihydrochloride	5·0 × 6	6–8	—	Cured†
	5·0 × 6	7–8	—	Cured‡
	2·5 × 6	7–8	—	Cured§
	1·0 × 6	7–8	—	Cured‡
	1·0 × 6	5–9	16	Moderate
	1·0 × 6	6	14–15	Moderate
	0·5 × 6	6	11–12	Slight
	0·5 × 6	6	17	Moderate
5-Aminohydroquinine hydrochloride	5·0 × 6	7–8	—	Cured‡
	5·0 × 6	7–8	11–12	Slight
	5·0 × 6	6	12–13	Slight
	1·0 × 6	7–8	12–13	Slight
	0·5 × 6	6–8	7–8	None
Quinidine hydrochloride	10·0 × 6	7–9	22–26	Moderate
	5·0 × 6	7–8	26	— ‖
	5·0 × 6	6–8	13	— ¶
	5·0 × 6	5–9	19–20	Moderate
Dihydroquinidine dihydrochloride	5·0 × 6	6–8	14–16	Moderate
Cinchonine hydrochloride	5·0 × 6	7–8	11–12	Slight
Dihydrocinchonine dihydrochloride	5·0 × 6	6–8	11–13	Slight
Cinchonidine hydrochloride	5·0 × 4 : 2·5 × 2	7–8	16–17	Moderate**
Dihydrocinchonidine hydrochloride	5·0 × 6	7–8	14–16	Moderate

* Re-inoculation on 24th day without effect. Re-inoculation on 46th day produced normal infection, indicating that bird had been cured.

† Re-inoculation on 30th day without effect. Re-inoculation on 40th day produced normal infection, indicating that bird had been cured.

‡ Re-inoculation on 26th day produced normal infection, indicating that bird had been cured.

§ Re-inoculation on 26th day without effect. Re-inoculation on 36th day produced normal infection, indicating that bird had been cured.

‖ Bird died on 26th day without showing evidence of infection.

¶ Bird died on 13th day without showing evidence of infection.

** Dose reduced owing to toxicity.

Cinchona alkaloids (Table I).

Five of these alkaloids have been compared in bird malaria by Giemsa, Weise and Tropp [1926] and the results of the two comparisons may be summarised as follows:

Drug	Giemsa, Weise and Tropp: Dose (mg.)	Giemsa, Weise and Tropp: Retardation of attack (days)	Present authors: Dose (mg.)	Present authors: Retardation of attack (days)
Quinine hydrochloride	2·5–3·0 × 4	12–13	2·0 × 6	8–9
Hydroquinine dihydrochloride	2·5–3·0 × 4	11	2·5 × 6	Cured
,, ,,	—	—	1·0 × 6	7–11 or cured
,, ,,	—	—	0·5 × 6	5–11
5-Aminohydroquinine (base)	2·5–3·0 × 4	11–12	—	—
5-Aminohydroquinine hydrochloride	—	—	5·0 × 6	4–7 or cured
,, ,,	—	—	1·0 × 6	5
Quinidine hydrochloride	1·25–1·5 × 4	12	5·0 × 6	14 or cured
Cinchonine hydrochloride	2·5–3·0 × 4	0–5	5·0 × 6	4–5

Giemsa, Weise and Tropp's results indicate that cinchonine is definitely inferior to any of the other four, which are all of about equal value, whilst our results show that hydroquinine is definitely superior to any of the other four, among which there is little to choose except that there are some signs that quinidine and aminohydroquinine, which both produced cures at a dose of 5 mg., may be better than quinine or cinchonine. From our results cinchonidine also appears to be as good as quinine. The only other investigation which need be mentioned on this point is that of Hegner, Shaw and Manwell [1928], who arrange four of these alkaloids in the following descending order as regards absorption by red blood-corpuscles, which they regard as an indication of efficiency against malaria. The figures are partition coefficients (concentration in corpuscles over concentration in serum) for chicken blood. Quinine, 5·0; quinidine, 4·4; cinchonine, 4·3; cinchonidine, 3·8.

The results of such comparative clinical trials as have been made with the principal cinchona alkaloids are equally confusing.

MacGilchrist [1915–16] arranges them in the following descending order:

$$\text{Hydroquinine} \longrightarrow \begin{matrix}\text{Cinchonine}\\ \text{Quinine}\\ \text{Quinidine}\end{matrix} \longrightarrow \text{Cinchonidine}$$

It is remarkable that notwithstanding MacGilchrist's results hydroquinine has not been given a more extended trial in human malaria. The results of our tests on birds show clearly that hydroquinine is much more active than quinine, and suggest that unless its action on human malaria is very different from its action on bird malaria hydroquinine should prove to be of great value in the treatment of the former disease.

Fletcher [1923] says that in doses of 10 grains, twice a day, quinine, quinidine, cinchonine and cinchonidine appear to be of equal value in bringing about the disappearance of malaria parasites, but that in small doses of 5 grains, twice a day, cinchonine did not appear quite so potent as quinine and quinidine, whilst cinchonidine was definitely inferior to the other three.

A comparison of quinine and quinidine made under the auspices of the

Medical Research Council [1925] resulted in the establishment of the practical equivalence of quinine and quinidine, and this view is accepted by the Malaria Commission of the League of Nations [1927], who state that quinine and quinidine are practically of equal value in doses of 1 g. and add that cinchonine is only equally effective in doses of 1·5 g.

Sinton and Bird [1929] on the contrary regard quinine, cinchonine and cinchonidine as of almost equal value in benign tertian malaria and quinidine as inferior to these three.

So far as other alkaloids in Table I are concerned, the results indicate that 5-aminohydroquinine is no better than hydroquinine and that whilst hydrogenation of quinine produces the more active hydroquinine, the hydrogenation of quinidine, cinchonidine and cinchonine does not enhance their antimalarial value. This last observation confirms clinical results recorded by Giemsa and Werner [1914].

It must, however, be pointed out that, so far as bird malaria trials are concerned, the simple technique employed in which a drug is tested on one bird is inadequate. This point is better brought out in Table II, but is also evident in the results recorded in Table I. Thus 5 mg. of quinine hydrochloride gave a retardation of only 5 days in the single bird to which it was given, a less powerful action than that sometimes produced by the smaller doses. A similar variability appears even when, as in Giemsa, Weise and Tropp's experiments [1926], three birds are used. In human malaria the response of patients to treatment with quinine is notoriously variable, a fact usually attributed to variable absorption of the drug from the intestine, and it is reasonable to suppose that birds also would show this variability. Less variable results might be obtained if the drugs could be administered by intramuscular or intravenous inoculation, but this procedure is not practicable for repeated doses of considerable amounts to small and delicate birds. Our experience shows that, using the technique adopted, larger numbers of birds must be used than is at present the custom if trustworthy results are to be obtained.

So far as comparative clinical trials are concerned, there is one point which calls for comment. With the exception of the comparison of quinine and quinidine, made under the auspices of the Medical Research Council, no particular pains seem to have been taken to ensure purity of the alkaloids used. Dawson and Garbade [1930] have recently called attention to this matter in another connection, and if the information they provide as to the extent of the contamination of commercial supplies of individual cinchona alkaloids is accurate, it may account for some of the discrepant results obtained by different observers. Thus, if cinchonidine is, as Dawson and Garbade state, liable to contain up to 10 % of quinine with some hydrocinchonidine, it is conceivable that variation in the amount of these impurities may account for the varying estimates of the antimalarial value of this alkaloid formed by different observers.

Table II. *Esters of quitenine.*

Drug	Dose in mg. No. of daily doses given	Day on which parasites appeared in blood: Control birds	Day on which parasites appeared in blood: Treated birds	Anti-malarial action
Methylquitenine monohydrochloride	10·0 × 6	6–8	11	Slight
	5·0 × 6	7–8	14–15	Slight
	5·0 × 6	6–8	7–8	None
Ethylquitenine monohydrochloride	10·0 × 6	6–8	7–8	None
	5·0 × 6	7–8	9	None
Ethylquitenine dihydrochloride	10·0 × 6	7–9	14–15	Slight
	5·0 × 6	7–8	6	None
n-Propylquitenine monohydrochloride	5·0 × 6	7–8	7–8	None
*iso*Propylquitenine monohydrochloride	10·0 × 6	6–8	12	Slight
	5·0 × 6	7–8	9	None
n-Butylquitenine monohydrochloride	4·1 × 6*	6–8	5	None
n-Butylquitenine dihydrochloride	10·0 × 6	6–8	9–10	Slight
	5·0 × 6	6–8	6	None
	5·0 × 6	6–8	9	Slight
n-Butylquitenine monoacetate	5·0 × 6	7–9	—	Cured†
	5·0 × 6	7–8	14–15	Moderate
	5·0 × 6	7–8	11–12	Slight
	1·0 × 6	7–9	7–8	None
*iso*Butylquitenine dihydrochloride	5·0 × 6	7–8	7–8	None
	5·0 × 6	7–8	12–13	Slight
n-Amylquitenine dihydrochloride	5·0 × 6	7–8	16–17	Moderate
	1·0 × 6	6–11	6	None
*iso*Amylquitenine monohydrochloride	5·0 × 6	7–9	—	Cured†
	5·0 × 6	7–8	10–11	Slight
	5·0 × 6	7–8	9–10	Slight
	1·0 × 6	7–9	10	Slight
*iso*Amylquitenine ethylsulphonate	5·0 × 6	5–9	12–15	Slight
Benzylquitenine monohydrochloride	2·5 × 6*	6	7–8	None
Benzylquitenine dihydrochloride	5·0 × 6	7–8	14–16	Moderate
Benzylquitenine ethylsulphonate	5·0 × 6	6–8	14–15	Moderate
n-Octylquitenine dihydrochloride	2·5 × 6	7–8	7–8	None
Esters of cinchotenidine.				
Ethylcinchotenidine monohydrochloride	5·0 × 6	7–8	12–13	Slight
	1·0 × 6	7–8	5	None
*iso*Amylcinchotenidine monohydrochloride	5·0 × 6	7–8	7–8	None
	5·0 × 6	7–8	14–15	Moderate
Esters of quitenidine.				
Ethylquitenidine monohydrochloride	10·0 × 6	7–9	9	None
*iso*Amylquitenidine dihydrochloride	5·0 × 6	7–8	10	Slight
Esters of cinchotenine.				
Ethylcinchotenine monohydrochloride	10·0 × 6	6	10	Slight
*iso*Amylcinchotenine monohydrochloride	5·0 × 6	7–8	7–8	None
*iso*Amylcinchotenine dihydrochloride	5·0 × 6	7–8	6	None
	5·0 × 6	7–8	7–8	None

* Maximum possible dose owing to low solubility.
† Re-inoculation on 26th day caused normal infection, indicating that bird had been cured.

Quitenine series (Table II).

It has been clearly established already that the free acid itself—quitenine—is inactive both in bird malaria and in human malaria [Stephens *et al.*, 1919], and the object of examining the series of esters of quitenine and the analogous derivatives of the other three cinchona alkaloids dealt with in Table II was to confirm the observation of Giemsa, Weise and Tropp [1926] that the ethyl ester of quitenine is active, and to ascertain whether, by the use of higher alcohols, more active alkylquitenines could be obtained.

The results recorded in Table II make it clear that quitenine does regain activity on esterification and that, on the whole, activity increases as the homologous series is ascended, reaching its maximum at butyl or amyl, at which point cures begin to appear. But, apart from these occasional cures, none of these esters appears to approach hydroquinine or even quinine and quinidine in activity, and the simple quitenine esters do not appear to hold out any hope of a considerable improvement on quinine.

It has been pointed out already by Schnitzer [1926] that esters of quitenine act as local anaesthetics. Dr J. Trevan has kindly tested this point for us. He finds that the esters do act as local anaesthetics, the benzyl ester being markedly active, but that they are too irritating to be of practical value.

We have also examined the esters of the carboxylic acids analogous to quitenine derived from the other three cinchona alkaloids, viz. cinchotenidine from cinchonidine, quitenidine from quinidine and cinchotenine from cinchonine. In these three cases only the ethyl and *iso*amyl esters were used. Here also it is clear, though at most two birds only were used in the trial of each substance, that the esters are active, the best of them being *iso*amyl-cinchotenidine.

Other drugs with a reputation for antimalarial action.

Alstonia species. Two of the authors have at present under chemical examination the following species of *Alstonia*:

A. congensis	West Africa.
A. scholaris	India and Philippine Islands.
A. macrophylla	Philippine Islands.
A. constricta	Australia.

The first two contain the alkaloid echitamine [Goodson and Henry, 1925]. The third and fourth contain no echitamine, but are rich in alkaloids, which are still under examination. The total alkaloids of *A. scholaris* showed perhaps a slight action in doses of 2·5 mg., which was the highest dose tolerated, and those of *A. constricta* in doses of 10 mg., but not in doses of 5 mg. Echitamine sulphate was slightly active at a dosage of 5 mg. The total alkaloids of *A. macrophylla* and *A. congensis* were inactive.

Picralima klaineana. The seeds of this plant are regarded by natives in Northern and Western Africa as a cure for malaria. The principal alkaloid present, akuammine [Henry and Sharp, 1927], and the total alkaloids, used as hydrochlorides, proved to have no action in bird malaria.

Nectandra rodioei bark. This material (greenheart bark) is used in British Guiana and elsewhere as a remedy for malaria. The total alkaloids, used as hydrochlorides, had no action in bird malaria.

Indole alkaloids. The alkaloids, echitamine and akuammine, referred to above, are probably both indole derivatives; the former is slightly active. Harmine and harmaline were considered by Gunn and Marshall [1920] to be active in human malaria. Both were tried in this series of experiments and found to be inactive in bird malaria in doses of 2·5 or 5·0 mg.

iso*Quinoline derivatives.*

Among the alkaloids, which have at one time or another been used for the treatment of malaria, is narcotine, which belongs to the *iso*quinoline group. It seemed worth while, therefore, to try a series of *iso*quinoline alkaloids in bird malaria and the following were used.

Papaverine hydrochloride
Tetrahydropapaverine hydrochloride
Pavine hydrochloride
N-Ethyltetrahydropapaverine hydrochloride
N-Methylpavine hydrochloride
N-Methylpavine methochloride
Laudanosine hydrochloride
Aminolaudanosine hydrochloride
Berberine phosphate
Anhydromethylcanadine

None of these had any traceable preventive or curative action. It has been suggested by Waldorp [1926] that berberine is a useful drug for provoking latent malaria into activity, but no such effect was observed in these experiments.

Miscellaneous drugs.

Hegner, Shaw and Manwell [1928] have already tried a large number of miscellaneous drugs in bird malaria without finding anything outside the cinchona alkaloid series which seemed promising, with the possible exception of one organic mercury compound.

In spite of these discouraging results, it was considered worth while to try a number of compounds, which we had available. These were: pseudaconine, conessine, daphnandrine, acriflavine, proflavine, sparteine.

There was no reason to expect protozooicidal activity in the case of pseudaconine and daphnandrine, but conessine is now known to be very toxic to *Entamoeba histolytica*, and sparteine has long been supposed to resemble the cinchona alkaloids in possessing a quinuclidine structure. None of these six substances showed any activity in bird malaria.

SUMMARY.

1. Out of an extensive series of alkaloids tested on canaries infected with *Plasmodium inconstans* only the alkaloids belonging to, or derived from, the cinchona series showed marked remedial action.

2. Of the natural cinchona alkaloids the most active was hydroquinine followed by quinidine, quinine, cinchonidine and cinchonine in descending order, though there is little to choose among the last four. Hydrogenation of quinidine, cinchonidine and cinchonine does not appear to lead to increased activity, as it does in the case of quinine.

3. Activity is restored to quitenine and the analogous carboxylic acids derived from quinidine, cinchonidine and cinchonine, by esterification, and there are clear indications of a rise in activity as the molecular weight of the alcohol used for esterification is increased, which reaches a maximum at butyl- or amyl-quitenine.

4. Among other alkaloidal drugs having some reputation as remedies for malaria, activity was observed only in two *Alstonia* species, viz. *A. scholaris* and *A. constricta*, and there it was slight. The former species no doubt owes its activity to echitamine, which exhibited slight action in doses of 5 mg.

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XCIX. THE INACTIVATION OF PANCREATIC LIPASE BY HEAT.

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Very little is known about the heat-inactivation of pancreatic lipase, and the work that has been done is rather qualitative than quantitative. Terroine [1910] observed that pancreatic secretion was very sensitive to heat and rapidly lost its lipolytic activity at 45°. At 65° the lipolytic activity was completely lost in 10 minutes. According to the same author bile salts accelerated this process. Terroine also investigated the effect of activating the trypsin in the secretion with enterokinase, and observed that the lipase was then more rapidly destroyed. The addition of coagulated egg-albumin decreased the action of the trypsin-kinase on the lipase. Mellanby and Woolley [1914] have observed that pancreatic secretion, of alkalinity 0·12 *N* sodium carbonate, lost its lipolytic activity at the rate of 10 % per hour at 40°. At 50° the rate was 50 % per hour and at 60° the whole of the lipolytic activity was lost within 5 minutes. The material was found to lose its activity at a slightly slower rate when the solution was neutral. When the solution was made 0·02 *N* with respect to HCl it was found to be destroyed very rapidly. These authors also observed that the rate of inactivation was increased when enterokinase was added, and that serum-albumin and egg-albumin protected the lipase from destruction. Lombroso [1914] observed that in the presence of such substances as glycerol, glucose, sucrose and maltose (compounds which are heavily hydrated in solution) pancreatic lipase was much more stable to heat. Willstätter and Waldschmidt-Leitz [1923] employed glycerol as the most efficient stabiliser of pancreatic lipase in their experiments on the purification of this enzyme. The work described in this paper deals with a quantitative investigation of the heat-inactivation of pancreatic lipase.

The estimation of pancreatic lipase.

It is necessary in the first place to have some reliable method for the estimation of the lipase. The materials employed in the estimation were prepared as follows.

(*a*) *Glycerol extract of lipase.* Dried gland powder was prepared by drying pig's pancreas with acetone and ether according to the procedure of Willstätter

and Waldschmidt-Leitz [1923]. 6 g. of this dried gland powder were added to 100 cc. glycerol-water mixture (containing 80 % glycerol), the mixture then being kept with occasional shaking at 30° for 4 hours. After centrifuging, the supernatant liquid was clarified by filtration through two layers of filter paper. The clear liquid thus obtained is referred to as a "glycerol extract of lipase."

(*b*) *Neutral olive oil.* The method employed was a modification of that due to Corran and Lewis [1928]. Commercial olive oil (Boots's finest Lucca olive oil) was shaken with an equal volume of 1 % sodium hydroxide solution in a separating funnel at about 75°. After standing, the aqueous layer was separated, and the emulsion of oil remaining washed with hot distilled water until all soap was removed. The oil was then dried by heating on a water-bath at 2 mm. pressure. The same stock commercial olive oil was employed throughout, and the saponification number 192·5 was found to be unaltered in different preparations[1].

(*c*) *Egg-albumin solution.* 2 g. of B.D.H. egg-albumin powder were dissolved in excess ammonia, the ammonia then being evaporated by impinging a stream of warm air (30°) on the surface of the liquid. The solution was left overnight in a vacuum desiccator containing sulphuric acid and then made up to 100 cc. It is referred to as 2 % albumin solution. The p_H of such a solution was found to be approximately 7·6.

(*d*) *Calcium chloride solution.* A solution of calcium chloride was made from pure fused calcium chloride free from calcium hydroxide. The solution was adjusted to contain 1 % calcium chloride by estimating the Cl ion concentration and diluting the solution accordingly.

(*e*) *Sodium chloride solution.* A 0·1 *N* solution of sodium chloride was prepared from B.D.H. sodium chloride which had been purified by precipitation from a saturated solution with HCl gas and dried at 105° for 3 hours.

(*f*) *Buffer solution.* The buffer described by Willstätter, Waldschmidt-Leitz and Memmen [1923] was made up by mixing 66 parts of *N* ammonia and 134 parts of *N* ammonium chloride. The p_H of this solution was measured with the glass electrode and found to be 8·9 at 30°.

Willstätter, Waldschmidt-Leitz and Memmen [1923] have observed that calcium chloride and egg-albumin are excellent activators of pancreatic lipase in an alkaline medium and employ these substances in their method of estimation.

The writer has verified the excellent activations of pancreatic lipase obtained with calcium chloride and egg-albumin, and numerous experiments on the combined action of the two activators have been carried out. The combined effect of the two was found to be slightly greater than the sum of the two separate effects, again confirming the results of the above authors. The experiments were carried out as described by these investigators. The enzyme

[1] The writer is indebted to Mr Priestman, of the Department of Industrial Chemistry, the University of Liverpool, for these determinations.

material was diluted with sufficient water to keep the total volume of the water solution 13 cc. 2·5 g. olive oil, 2 cc. buffer solution and the calcium chloride solution were added and the mixture was gently shaken. Finally, the solution of egg-albumin was added and the mixture shaken vigorously by hand for 3 minutes and then placed in a thermostat at 30° for 57 minutes. The contents of the reaction vessel were washed into an Erlenmeyer flask with 96 % alcohol so that the volume of the alcoholic liquid was 125 cc. 20 cc. ether were added and the acid titrated with 0·1 *N* alcoholic NaOH in the presence of thymolphthalein. Control experiments in which the buffer and protein of the enzyme material were titrated were also carried out to determine the degree of hydrolysis of the olive oil. A few of the results are given in the table below.

Table I. *Temp. 30°. Enzyme extract 0·4 cc. Total volume of water solution 13 cc.*

mg. $CaCl_2$	mg. albumin	Hydrolysis in cc. 0·1 *N* NaOH	mg. $CaCl_2$	mg. albumin	Hydrolysis in cc. 0·1 *N* NaOH
0	0	6·5	0	10	8·2
10	0	10·95	15	10	14·4
10	10	12·8	20	10	16·4
10	15	13·6	0	20	9·5
10	20	14·6	20	20	17·1
10	40	15·4			

In the presence of a high concentration of calcium chloride (greater than 15 mg.) a thick creamy mass, which contained all the oil, separated out when the shaking was stopped, and the reproducibility of the experiments was not so good as that obtained with smaller concentrations of calcium chloride, in which case good emulsification of the oil resulted. In general, the writer considered 10 mg. calcium chloride and 20 mg. egg-albumin in a total volume of 13 cc. water solution to be a good combination of activators to employ to swamp the effect of the substances accompanying the enzyme.

An investigation of the heat-inactivation of pancreatic lipase would necessarily involve the use of glycerol extracts. This might mean a considerable variation in the concentration of the glycerol in the determination mixture during such an investigation (*e.g.* only 0·2–0·4 cc. of a fresh glycerol (80 % glycerol) extract are sufficient to give a suitable hydrolysis, whereas after heating at 55° for 30 minutes 1·0–1·5 cc. are required to give the same hydrolysis). Willstätter, Waldschmidt-Leitz and Memmen [1923] observed that glycerol accelerated the action of pancreatic lipase under the conditions of the experiments, *i.e.* an initial p_H of 8·9. They, however, only worked with 0·2–0·4 cc. of an 87 % glycerol extract, and the control of the glycerol concentration was unnecessary. The writer has investigated the effect of glycerol on the action of pancreatic lipase in the absence of other activators and also in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. The glycerol employed was B.D.H. (A.R.) glycerol. The results are given in Tables II and III.

Table II. *The effect of glycerol in the absence of other activators. Temp. 30°. Enzyme extract 0·4 cc. Initial* $p_H = 8·9$. *Total volume water solution = 13 cc.*

cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH	cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH
0	6·3	3·0	15·5
1·0	9·4	4·0	17·8
2·0	12·1	5·0	19·2

Table III. *The effect of glycerol in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. Temp. 30°. Enzyme extract 0·2 cc. Initial* $p_H = 8·9$. *Total volume of water solution = 13 cc.*

cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH	cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH
0	15·3	3·0	16·5
1·0	16·0	4·0	16·6
2·0	16·3		

(The extracts employed in Tables II and III are similar but not identical.) It will be seen that the action of glycerol is not so marked in the presence of calcium chloride and albumin as when these activators are absent. Similar results were obtained when different enzyme concentrations were employed. Although the effect in the presence of calcium chloride and albumin is small, it was considered necessary to keep the concentration of the glycerol constant in a mixture for the determination of lipase. The quantity chosen was 2 cc. of pure glycerol. This quantity allows for the use of 4 cc. of a 50 % glycerol extract without bringing in any error due to the acceleration produced by uncompensated glycerol concentration.

Another factor which must be considered is the method of regulating the p_H when carrying out the heat-inactivation of lipase at different p_H values. By employing NaOH and HCl a wide range of p_H values may be obtained. The p_H in the method of estimation of the lipase proposed to be employed is essentially a changing one, the 2 cc. of buffer employed to give an initial p_H of 8·9 being insufficient to keep the p_H constant. It is therefore to be expected that the estimation will not give true values for the amount of lipase if the enzyme material contains large varying amounts of NaOH or HCl. (The protein in the extracts, especially in the water extract, makes it necessary to employ a considerable quantity of acid or alkali to alter the p_H appreciably, *e.g.* 1 cc. water extract of p_H 5·93 requires 2·1 cc. 0·1 *N* NaOH to bring the p_H to 9·5.) If the NaOH or HCl were neutralised prior to the actual process of estimation a certain quantity of NaCl would be present. It is therefore necessary to carry out experiments to see if NaCl has any effect on the action of lipase upon olive oil in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. The results are given in Table IV.

The effect is sufficient to necessitate the use of a constant concentration of NaCl in the estimation mixture. The quantity chosen was 1 cc. of 0·1 *N* sodium chloride in a total volume of 13 cc. water solution.

Table IV. *Initial* $p_H = 8{\cdot}9$. *Enzyme extract 0·3 cc. Temp.* = 30°. *Total volume water solution* = 13 *cc.*

cc. 0·1 *N* NaCl	Hydrolysis in cc. 0·1 *N* NaOH	cc. 0·1 *N* NaCl	Hydrolysis in cc. 0·1 *N* NaOH
0	17·7	0·75	18·3
0·25	17·85	1·0	18·4
0·5	18·15		

The conditions for the method of determination now being fixed it only remains to find a relation between the enzyme quantity and the degree of saponification.

The reactions were carried out in Erlenmeyer flasks of 50 cc. capacity fitted with tight-fitting rubber stoppers. Sufficient water and pure glycerol were added to the enzyme material to make the volume of the water 6 cc., and that of the glycerol 2 cc. 1 cc. 0·1 *N* NaCl, 2·5 g. olive oil, 2 cc. buffer and 1 cc. 1 % (10 mg.) calcium chloride were added and the mixture was shaken for a suitably short time (5 secs.). 1 cc. of 2 % (20 mg.) egg-albumin solution was then run in, and the mixture vigorously shaken by hand for 3 minutes. The reaction flasks were then placed in a thermostat at 30° for 57 minutes and tested. The contents were washed into Erlenmeyer flasks of 300 cc. capacity with 96 % alcohol so that the volume of the alcoholic liquid was 125 cc. 20 cc. ether were added and the acid was titrated with 0·1 *N* alcoholic NaOH in the presence of thymolphthalein as indicator. The degree of hydrolysis obtained with different quantities of enzyme material, under the above conditions, was investigated and a graph plotted showing the relation between the degree of saponification and the quantity of enzyme employed.

The results, given in Table V, are means of several readings.

Table V.

Enzyme expressed in cc. glycerol extract	Hydrolysis in cc. 0·1 *N* NaOH	Enzyme expressed in cc. glycerol extract	Hydrolysis in cc. 0·1 *N* NaOH
0·05	9·70	0·25	18·25
0·10	13·80	0·30	19·1
0·15	15·25	0·40	20·75
0·20	17·1	0·50	21·60

All enzyme extracts are not of the same activity, hence one does not obtain the same hydrolysis by using the same quantity of different extracts. For purposes of standardisation Willstätter and Kuhn [1922] have suggested an arbitrarily chosen measure which is termed an enzyme unit. The writer in adopting this method defines a lipase unit as the quantity of enzyme which produces a saponification of 2·5 g. of olive oil, equivalent to 20 cc. 0·1 *N* sodium hydroxide, in 1 hour at 30° under the conditions specified above. The adoption of this unit does not mean, however, that a given enzyme extract

containing say 0·5 unit will produce just half the amount of saponification in 1 hour at 30°. The actual degree of saponification produced by 0·5 (or any other submultiple or multiple) of a unit has to be determined by experiment. This is effected in principle by starting with a given quantity of extract, and finding by trial what volume of this extract contains just one lipase unit as defined above. Aliquot portions of this amount of extract are then examined for their saponifying powers, and a graph constructed connecting number of units with cc. of alkali required (Fig. 1). Using extracts from different glands, and proceeding in the above manner, a series of identical curves was obtained, the reproducibility being satisfactory.

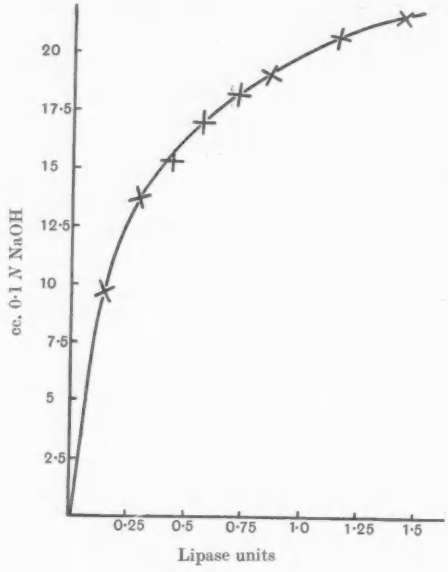

Fig. 1.

It may be mentioned that the unit as defined above is necessarily different from that of Willstätter, Waldschmidt-Leitz and Memmen, since the conditions of the experiment are different; the shape of the curve is not the same as that obtained by these authors.

The heat-inactivation of purified lipase.

(*a*) *Preparation of purified lipase.*

In order to obtain a purified lipase the writer has employed a modification of the method of Willstätter and Waldschmidt-Leitz [1923], who obtained lipase free from trypsin and amylase by carrying out two successive adsorptions on the aluminium hydroxide B of Willstätter and Kraut [1923]. They

observed that a mixture containing 57 parts by volume of 1 % diammonium phosphate, 3 parts of *N* ammonia and 40 parts of 87 % glycerol was most suitable for eluting the enzyme from the alumina-lipase adsorption complex. It was found impossible to expel the inorganic salts by dialysis against distilled water, as the lipase was completely destroyed during the process.

The method employed by the writer was as follows. Two successive adsorptions were carried out in exactly the same way as described by Willstätter and Waldschmidt-Leitz. After the second adsorption, however, the lipase was eluted from the alumina-enzyme adsorption complex with a mixture of 40 parts of glycerol, 3 of *N* ammonia and 57 of water. The ammonia was then evaporated *in vacuo* at 25°, and the solution placed in a desiccator over sulphuric acid, the p_H being thus brought to 7·0. This solution adjusted to contain 50 % glycerol was employed as a purified lipase. It was found to be free from trypsin-kinase. The yield was not as good as that obtained with the mixture containing ammonium phosphate, about 20 % of the lipase of the original glycerol extract being obtained. The absence of phosphate, however, is desirable in the present instance.

(*b*) *Apparatus employed.*

The apparatus employed is shown in Fig. 2.

The reaction vessel *A* is a silica vessel of about 70 cc. capacity, closed by a rubber bung *B*. *C* is a tube through which samples are withdrawn from time to time. Air enters the vessel *A* by means of the side arm *F*, which leads from vessel *G*, which contains the same solution as that under investigation. Air entering this vessel does so by way of a vertical tube *H* which dips under the surface of the liquid. Both vessels are kept well immersed in the water of the thermostat which is regulated to $\pm$ 0·05°. This apparatus prevents any change of concentration due to evaporation during an experiment.

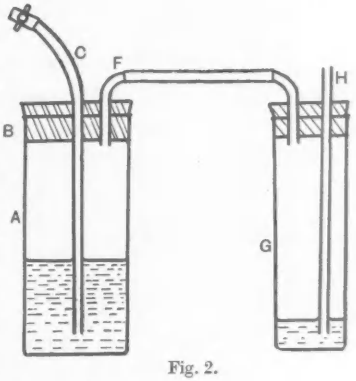

Fig. 2.

A definite quantity of lipase extract is taken and the p_H regulated by the addition of NaOH or HCl of known strength. This solution is placed in vessel *A* which is already at the temperature of the thermostat. Portions of the extract are withdrawn at various intervals of time and run into test-tubes standing in crushed ice. The first withdrawal is made 2–3 minutes after placing the extract in the thermostat. The lipase content of the various portions is estimated according to the method already described.

(c) *Course of the reaction.*

The applicability of the unimolecular expression to the heat-inactivation of this preparation of pancreatic lipase was tested by numerous experiments. The general behaviour is illustrated by Table VI.

The values of k_{uni} were calculated from the expression

$$k_{uni} = \frac{1}{t} \log_e \frac{a}{a - x},$$

where t = time in minutes, a = lipase units per cc. at zero time and $a - x$ = lipase units per cc. at time t.

Table VI.

Time in mins.	Lipase units/cc.	$k_{uni} \times 10^2$
Temp. 50°; 50 % glycerol; $p_H = 6{\cdot}00$		
0	0·365	
5	0·250	7·57
10	0·183	6·90
15	0·130	6·90
Temp. 40°; 50 % glycerol; $p_H = 8{\cdot}00$		
0	0·42	
30	0·29	1·23
60	0·225	0·90
90	0·15	1·14

It was observed that on the whole the constant decreased slightly in many of the experiments.

(d) *The effect of* p_H *upon the heat-inactivation.*

The effect of the p_H of the medium[1] is shown in Table VII and Fig. 3. The value for k_{uni} at a particular p_H is that obtained by taking the mean of several readings. It will be seen that the enzyme is most stable in the region of p_H 6·0.

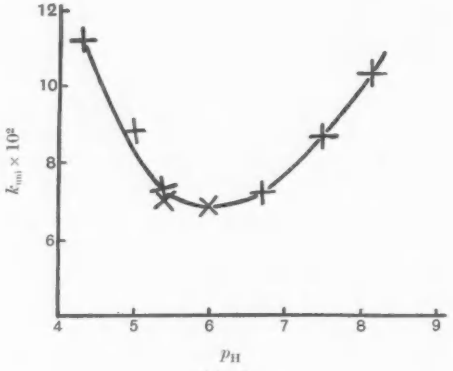

Fig. 3.

[1] The p_H measurements throughout this work were made by means of the glass electrode by the method described by Millet [1928].

Table VII.

Temp. 50°; 50 % glycerol

p_H	$k_{uni} \times 10^2$	p_H	$k_{uni} \times 10^2$
8·14	10·3	5·41	7·00
7·48	8·7	5·40	7·24
6·75	7·21	4·97	8·80
6·03	6·83	4·23	11·14

(*e*) *The critical increment of the process.*

In order to determine the critical increment for the heat-inactivation of purified pancreatic lipase, measurements were carried out at three different p_H values: (1) at the region of optimum stability, p_H 6·00; (2) at p_H 8·01; and (3) at p_H 5·00.

The results are given in Tables VIII, IX, X. The critical increment was calculated by substituting the values of k_{uni} into the integrated form of the equation

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}.$$

Table VIII.

50 % glycerol; $p_H = 6·0$

Time in mins.	Lipase units/cc.	$k_{uni} \times 10^3$
	Temp. 40°	
0	0·475	
60	0·315	6·83
120	0·21	6·80
180	0·145	6·59
		Mean = 6·74
	Duplicate exp.	Mean = 6·84
		Average = 6·80
	Temp. 50°	
0	0·365	
5	0·250	75·7
10	0·183	69·0
15	0·130	69·0
		Mean = 71·2
	Duplicate exp.	Mean = 70·7
		Average = 70·8

From these values of k_{uni} we arrive at the value of $E = 46{,}000$ calories at p_H 6·00.

Table IX.

Glycerol content of solution 50 %. $p_H = 8·01$.
Temp. 40°. Mean value of $k_{uni} = 1·14 \times 10^{-3}$.
Temp. 50°. Mean value of $k_{uni} = 10·1 \times 10^{-3}$.

From the above values of k_{uni} at 40° and 50° the value for the critical increment is found to be 44,000 calories at p_H 8·01.

Table X.

Glycerol content of solution 50 %. $p_H = 5{\cdot}1$.
Temp. 40°. Mean value of $k_{uni} = 7{\cdot}95 \times 10^{-3}$.
Temp. 50°. Mean value of $k_{uni} = 7{\cdot}80 \times 10^{-3}$.

The critical increment calculated from these values is 45,800 calories at p_H 5·1.

Table XI. *The critical increment in 25 % glycerol.*

Glycerol content of solution 25 %. $p_H = 6{\cdot}00$.
Temp. 40°. Mean value of $k_{uni} = 7{\cdot}90 \times 10^{-3}$.
Temp. 50°. Mean value of $k_{uni} = 8{\cdot}60 \times 10^{-3}$.

The critical increment calculated from these data is 47,800 calories.
The results obtained with purified lipase are summarised in Table XII.

Table XII. *Purified lipase.*

Glycerol concentration %	p_H	E in calories
50	6·00	46,000
50	8·01	44,000
50	5·1	45,800
25	6·00	47,800

Before discussing the results obtained above a very brief statement of the experiments carried out with unpurified lipase will be given.

The heat-inactivation of unpurified lipase.

Experiments were carried out with unpurified extracts of lipase in order to compare the results with those obtained with the pure enzyme.

These extracts were prepared by extracting 6 g. of the dried gland powder with 100 cc. of water or water-glycerol mixture for 2–4 hours at 30°. (Water gives a good extraction in 2 hours, while 80 % glycerol requires a longer time.) The mixture was then centrifuged and the supernatant liquid clarified by filtration.

In 80 % glycerol it was found that the course of the reaction follows the unimolecular expression quite satisfactorily except at p_H values on the acid side of the p_H of optimum stability ($p_H = 6{\cdot}0$) (Table XIII). In attaining these p_H values by the addition of HCl a precipitate was formed. (No precipitate was formed on adding NaOH.) This precipitate would carry along with it some of the lipase, and presumably this portion of the lipase is less liable to inactivation. At $p_H = 5{\cdot}0$ the velocity recorded is the initial value (since in this case the k falls with time).

Table XIII.

Temp. 50°; 80 % glycerol.

p_H	$k_{uni} \times 10^3$	p_H	$k_{uni} \times 10^3$
5·00	26·05	8·20	34·3
5·99	7·18	9·20	48·8
7·20	17·26	10·23	162·8

The results show that the enzyme is most stable in the region of p_H 6·0, as was the case with the purified enzyme in 50 % glycerol.

In the case of the water extract the unimolecular constant was found to decrease markedly with time at all p_H values. Thus at 40° and p_H 5·93 the "constant" decreased 50 % in 90 minutes. This decrease in the constant is more marked at 50°, while at 30° the decrease in 60 minutes only amounts to 15 %. The extract was most stable in the region of p_H 6·0.

The critical increment for the process was determined by measuring k_{uni} at different temperatures both in the presence and in the absence of glycerol. In the case of water extracts the initial values of k_{uni} were used to calculate the critical increment.

The results are summarised in Table XIV.

Table XIV. *Unpurified lipase.*

Glycerol conc. %	p_H	Temp. interval in ° C.	E in calories
80	5·99	45–50	101,000
80	5·99	50–55	103,000
80	9·30	45–50	92,000
50	5·98	45–50	57,500
0	5·93	30–40	35,000

Discussion of results.

In agreement with the results obtained for other enzymes the p_H of optimum stability of purified pancreatic lipase does not coincide with its "optimum p_H," *i.e.* p_H of optimum activity upon a substrate. The optimum thermal stability is in the region of p_H 6·0, whereas the "optimum p_H" is 8·0. The same behaviour is exhibited by the unpurified material.

In general it may be said that the behaviour of the purified extract is regular and reproducible. On the other hand, in the absence of glycerol the behaviour of the unpurified extract would depend upon the amount of trypsin-kinase present. The trypsin-kinase content of the unpurified material was determined according to the method of Willstätter *et al.* [1926], but without activation with enterokinase. It was found that a water extract of the "dried gland powder" contained 1·97 trypsin units per cc. and the 80 % glycerol extract 0·48 trypsin units per cc.[1]

Willstätter and Persiel [1924] have shown that glycerol inhibits the action of trypsin-kinase, hence this proteolytic enzyme would not be expected to act in 80 % glycerol. The observations of Terroine [1910] and Mellanby and Woolley [1914] indicate that trypsin activated with enterokinase (*i.e.* trypsin-kinase) destroys lipase. During the process of heat-inactivation of an impure water extract the writer has observed that a small amount of acid is formed, whereas in the 80 % glycerol extract no measurable amount results. In an 80 % glycerol extract, as would therefore be expected, the process of heat-

[1] The writer is indebted to Mr Pace, of the Department of Physical Chemistry, University of Liverpool, for these determinations.

inactivation, even of the unpurified material, is unimolecular. In a water extract, however, there are two processes going on: (1) the destruction of the lipase by heat; (2) the destruction of the lipase by trypsin-kinase. A unimolecular velocity constant for heat-inactivation is therefore not to be expected in this case.

It is also suggested that the change in the critical increment for the heat-inactivation of unpurified lipase with change in glycerol content is due to the action of trypsin-kinase, this enzyme destroying the protein carrier and thereby inactivating the active grouping of the lipase. In 80 % glycerol the trypsin-kinase effect is minimal and the observed critical increment of the heat-inactivation of unpurified lipase is very large—of the order 90,000–100,000 calories. In water the trypsin-kinase effect ought to be maximal. The critical increment of hydrolysis of caseinogen by trypsin-kinase is of the order 14,000 calories [Moelwyn-Hughes, Pace and Lewis, 1930]. This low value is probably the reason why the apparent critical increment of heat-inactivation of unpurified lipase in water is as low as 35,000 calories.

The most interesting observation is the relatively low value for the heat-inactivation of purified lipase, namely, 46,000 calories. As this value is obtained in 50 % and 25 % glycerol it seems reasonable to assume that much the same value holds in water. This value should be compared with 90,000–100,000 calories obtained for the unpurified lipase, under conditions in which the trypsin-kinase is ineffective (*i.e.* in 80 % glycerol). The effect of purification is therefore to reduce the critical increment to approximately one-half. This naturally accounts for the fact that the purified material is much less stable than the unpurified. At the same time, one is at a loss to explain precisely the manner in which this reduction in critical increment has been brought about by the act of purification. The purification consists mainly in the removal of protein, and this change in the accompanying substances in some way causes a lowering in the critical increment[1]. It is interesting to note that the critical increment of purified lipase is of the same order as that obtained by Pace [1930] for the heat-inactivation of trypsin itself, free from enterokinase and the pre-stage of this activator, the value obtained being 40,000 calories.

[1] The unpurified lipase has protective impurities adsorbed or otherwise combined with it. The extent of such adsorption will be a function of temperature, adsorption in general diminishing as temperature rises. Hence at the higher temperature the unpurified lipase is now partly purified by removal of impurity and hence is thermally inactivated at a greater rate than would have been found had the extent of adsorption remained independent of temperature. It follows that a correspondingly high value (90,000–100,000 cals.) is obtained for the apparent critical increment. If this point of view be the correct one, it may be expressed in a quantitative form. Thus if Q is the (positive) heat evolved in the adsorption of the impurities on one "molar" unit of enzyme, E_0 the true critical increment of heat-inactivation of purified enzyme and E_{obs} the observed value for the unpurified enzyme, then we would expect

$$E_{obs} = E_0 + Q.$$

Setting $E_{obs} = 95{,}000$, $E_0 = 45{,}000$ cals., it follows that $Q = 50{,}000$ cals. This is a very high value for adsorption but it has to be remembered that the "molecular unit" of an enzyme is exceedingly large and the number of molecules of adsorbed material per enzyme unit correspondingly great.

SUMMARY.

1. A modification of the method of Willstätter, Waldschmidt-Leitz and Memmen for the estimation of pancreatic lipase has been employed in investigating the heat-inactivation of this enzyme. The relation between the concentration of the enzyme and the degree of hydrolysis has been obtained under the new conditions, and a lipase unit, necessarily different from that of the above authors, has been defined and employed in the later work described.

2. A purified lipase has been prepared by two adsorptions on aluminium hydroxide. In order to obtain a preparation free from salts an elution mixture of glycerol, water and ammonia is employed in eluting the enzyme after the second adsorption.

3. Experiments have been carried out on the heat-inactivation of this purified lipase. The course of the heat-inactivation process was found to be unimolecular.

4. The effect of p_H upon the heat-inactivation of lipase prepared in the above manner has been investigated. It has been found that the optimum stability of the lipase is about p_H 6·0.

5. The critical increment for the heat-inactivation process in 50 % glycerol has been determined at three p_H values: 6·0, 8·01 and 5·0. It was found that the critical increment was sensibly the same at all three points, and was of the order of 46,000 calories.

6. The critical increment was likewise determined in 25 % glycerol and p_H 6·0 and was found to be 47,800 calories. It is concluded therefore that in the absence of glycerol the critical increment would be of this order.

7. Experiments were also carried out on the heat-inactivation of extracts of unpurified lipase. The course of the reaction in 80 % glycerol was found to be unimolecular, whereas in the water extract of the "dried gland powder" the unimolecular constant was found to decrease with time. This decrease is explained by the action of the trypsin-kinase in the water extract.

8. The effect of p_H upon an 80 % glycerol extract and water extract of unpurified lipase has been investigated. It was found that the enzyme is most stable at about p_H 6·0.

9. At p_H 6·0 and in 80 % glycerol the critical increment for the process in unpurified extracts is shown to be of the order of 100,000 calories, whereas in 50 % glycerol the value is 57,500 calories, and in water 35,000 calories. This change in the critical increment with change in the concentration of glycerol is ascribed to the action of the trypsin-kinase. The critical increment for the heat-inactivation process of an unpurified extract containing 80 % glycerol at p_H 9·3 is of the order 92,000 calories, which is sensibly the same as 100,000 calories.

10. The fall from 95,000 calories for unpurified lipase to 45,000 calories for purified lipase is in agreement with the increased instability of the purified material.

The work described in this paper was carried out under the direction of Prof. W. C. M. Lewis, to whom the writer is indebted for help and advice. The writer also wishes to thank the Trustees of the H. B. Webb Fund, of the University of South Africa, for the award of a Research Scholarship, and is indebted to Imperial Chemical Industries, Ltd., for a grant to the Department of Physical Chemistry of the University of Liverpool.

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C. CARBOHYDRATE METABOLISM OF NORMAL AND TUMOUR TISSUE.

PART I. A METHOD FOR THE MEASUREMENT OF THE RESPIRATORY QUOTIENT.

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Metabolic measurements upon isolated tissues *in vitro* have attained a high degree of accuracy in so far as the consumption of oxygen, or the measurement of glycolysis, alone is concerned. However, when the measurement of CO_2 actually formed in the tissues is considered, the existing methods are found to be generally unsatisfactory. Since the whole significance of the respiratory quotient is dependent upon very slight variations in its numerical value, it is of the utmost importance that the accuracy of the determination of CO_2 should be as nearly as possible equal to that of the oxygen measurement. The methods in common use are as follows.

(1) The oxygen consumption of a piece of tissue is measured manometrically, the CO_2 being absorbed by KOH. Comparison of the readings with those obtained with another piece of tissue in a second manometer vessel without KOH allows the CO_2 to be calculated [Büchner and Grafe, 1924]. A differential arrangement may also be used, as in Thunberg's respirometer [Fenn, 1927].

(2) In order to overcome the difficulties due to insufficient buffering and at the same time to use a physiological concentration of bicarbonate in the medium, Warburg introduced his "improved method" [Warburg, 1926]. This method is based on the different solubilities of oxygen and CO_2, and the respiration is calculated from readings made with two vessels with similar pieces of tissue but with different volumes of fluid in each. It is of great value for the measurement of aerobic glycolysis but cannot be used for the true respiratory quotient, since the figure for CO_2 obtained includes the "extra CO_2" liberated from the bicarbonate by the production of acid, in addition to the respiratory CO_2. Referring to the estimation of true respiratory quotient in bicarbonate-containing solutions, Warburg [1926] states: "On account of the high physiological bicarbonate concentration it is difficult to estimate the increase of bound CO_2 with sufficient accuracy, and it is questionable whether this difficulty can be overcome by a differential procedure."

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(3) Recently Crabtree [1929] has described a method in which the "improved method" of Warburg is combined with estimation of chemically bound CO_2 by acidification before and after the experiment. The objection quoted above greatly limits the accuracy of this method, since the change of bound CO_2 necessary for calculating the R.Q. appears as a small difference between two large readings.

(4) Analysis of the gas-phase in equilibrium with the solution containing the tissue is the basis of a further method. The application of the catharometer to the problem has been described by Slater [1926]. The apparatus is necessarily complicated.

(5) A method has been described by Fenn [1928] in which the CO_2 is absorbed by barium hydroxide solution in a special vessel arranged for the measurement of electrical conductivity. The total CO_2 liberated may be calculated from the alteration of conductivity. The oxygen is determined by Thunberg's principle.

Leaving aside method 2, which is unsuitable and, in fact, was not designed for the measurement of true respiratory quotient, a fundamental objection is found to apply to the above methods. This objection is that no allowance is made for the variations in the CO_2 chemically bound in the tissue or the surrounding solution. Any change of the acid-base equilibrium of the whole system, due to production or consumption of acids or bases by the tissue, leads to the diminution or increase of the amount of CO_2 chemically bound. Such changes are included in the figure for CO_2 production obtained by the methods 1, 4 and 5. In practice, quantitatively the most important part of the error caused by neglecting the chemically bound CO_2 is due to the lactic acid produced by glycolysing tissues. In many cases this error is so large that it makes the figure for CO_2 valueless. Many figures for respiratory quotient much above unity which may be found in the literature are obviously due to the glycolysis of the tissues examined.

From the methods described, only that of Crabtree is theoretically free from this objection. Here, however, as was mentioned above, other practical considerations limit the accuracy.

For the measurement of true respiratory quotient the only remaining principle is that in which buffer solutions almost free from CO_2 are used. The bound CO_2 present in the tissue and solution at the beginning and end of the experiment is liberated by addition of strong acid and is included in the calculation. On account of this important advantage the method has been used by many workers [Warburg *et al.*, 1914, 1921, 1924; Meyerhof, 1919; Gerard, 1927; Schorr, Loebel and Richardson, 1930; Richardson, Schorr and Loebel, 1930]. A detailed description of an experiment is given by Richardson [1929]. The solution used is buffered by phosphate instead of bicarbonate. The figures for CO_2 are obtained from manometric measurement on two pieces of tissue in two separate manometers, one with KOH, the other with acid in a side-bulb, so that the latter can be mixed with the solution and tissue in the

main part of the vessel at the end of the experiment. The vessel with KOH gives the oxygen respiration, and this, together with the change of pressure occurring in the second vessel, permits the calculation of the CO_2 produced. A third manometer vessel is used to determine by acidification the initial CO_2 bound by the tissue and solution. The CO_2 content of the solution itself is determined in yet another vessel.

Valuable as the method is in certain selected cases, it suffers from an important defect which applies to all the methods where the CO_2 is determined from different pieces of tissue, already described under headings 1, 2 and 3. This is the implicit assumption that the extent of respiration, *i.e.* the volume of O_2 consumed per hour per unit weight of tissue, is exactly the same in two slices of the same tissue. Although the effect of such a difference on the respiratory quotient is not usually very serious, it is, nevertheless, desirable to remove the source of error. This is particularly the case when tissues with small respiration are concerned, and prolonged experiments are, therefore, necessary.

Another difficulty is due to the uncertainty of the amount of CO_2 retained by the tissue and phosphate during the period of temperature-equalisation preceding the experiment. During this period a portion of the respiration CO_2 is retained by the phosphate. In addition to this objection, in the case of glycolysing tissues an uncertain amount of CO_2 is driven off by the lactic acid formed. The most serious point is that all of these errors concern only the CO_2 without compensatory influence on the oxygen values.

With the above points in view, a method has been worked out so that the actual measurement could be made on a single piece of tissue[1], only one correction—a simple determination of preformed CO_2—being necessary. The accuracy of the results is not affected by glycolysis.

Method.

Principle. Both oxygen consumed and CO_2 produced are determined by readings on the same manometer. The measurement is made on thin slices of tissue [Warburg, 1926] suspended in CO_2-free Ringer solution, buffered by means of phosphate and in equilibrium with pure oxygen. The CO_2 formed in respiration is absorbed by barium hydroxide solution. The diminution in pressure which occurs is used for the calculation of respired oxygen. After a definite time, an excess of strong acid is added, and the whole of the CO_2 is given up from the barium hydroxide, phosphate and tissue. The resulting positive pressure is used for the calculation of the total CO_2 at the end of the experiment.

This figure, corrected for the CO_2 content at the beginning of the experiment, gives the amount of CO_2 formed in respiration during the experimental period.

[1] The method described was worked out before that of Meyerhof and Schmitt [1929] came to our notice. Since their method, though based on similar principles, is solely concerned with muscle and nerve, the practical details in the two methods differ in many important points.

Apparatus[1]. The vessel (Fig. 1) used is attached by means of the ground joint to a Barcroft manometer. The total volume is about 20 cc. and is determined by weighing with mercury. The central portion *A* contains a piece of tissue suspended in 2 cc. phosphate-Ringer solution. The annular trough *B* contains 0·5 cc. of a cold-saturated solution of barium hydroxide. The side-bulb *C* contains 0·3 cc. of 2·5 *N* HCl, which can be tipped into the main compartments and the whole contents mixed when desired.

Solutions. Stock sodium phosphate solution. An isotonic solution of sodium dihydrogen phosphate and disodium hydrogen phosphate at p_H 7·4 is prepared

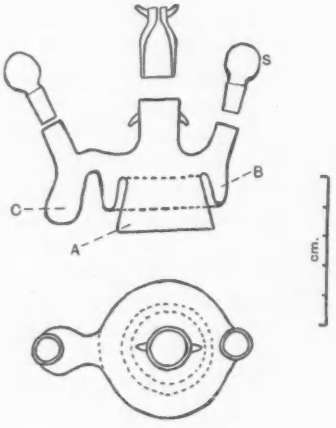

Fig. 1.

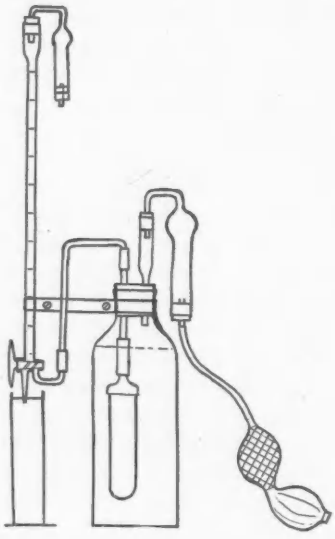

Fig. 2.

with the usual precautions to exclude CO_2. With the samples of dried phosphate used, the weights were 2·43 g. NaH_2PO_4 and 12·68 g. Na_2HPO_4 to 1 litre. Such a solution is about 0·109 *M* in total phosphate. This solution is stored in a burette protected from CO_2.

Phosphate-Ringer solution. The above solution is diluted for use by means of a suitable salt solution consisting of isotonic solutions of NaCl, $CaCl_2$ and KCl in the proportion 100: 2: 2 to which is added 10 % glucose solution to make the final concentration 0·2 %. The solution is boiled for 15 minutes and, after cooling in a stream of pure oxygen, is diluted to the original volume with CO_2-free water. The phosphate solution described above is then added. For tissues with little or no glycolysis 10 cc. phosphate per 100 cc. salt solution is

[1] The apparatus was made for us by Messrs C. Dixon & Co., 27 Devonshire Street, London, W.C. 1.

sufficient. For tumours, retina and other highly glycolysing tissues a more strongly buffered solution should be used. A mixture of 40 parts to 100 salt solution has a concentration of secondary phosphate equivalent to that of bicarbonate in the solution used by Warburg. It is particularly necessary to cool the solution very thoroughly before adding the phosphate, since the mixture is supersaturated with calcium phosphate. If the vessels are perfectly clean the solution usually remains quite clear.

Barium hydroxide solution. A cold saturated solution of barium hydroxide (*ca.* 0·3 *N*) is prepared and poured into the bottle of an automatic burette (10 cc.), fitted with a filtration candle (Berkefeld V) as in Fig. 2, CO_2-free air having been previously drawn through the whole system. The capacity of the bottle is *ca.* 500 cc. and the whole arrangement must be easily and quickly brought to the vessels when required.

Procedure. Three vessels are used in the example described (more if duplicate estimations are required. See protocol, p. 913). Another vessel, without tissue and containing the solutions only, is used as a thermobarometer to correct the readings for variations of temperature and barometric pressure during the experiment.

Preparation of the tissue. Thin slices are cut from the fresh tissue with the razor. As Warburg [1926] has shown, the thickness must not exceed a certain limiting value, in order to allow the adequate diffusion of oxygen, carbon dioxide and lactic acid in the tissue. The thin sections are rinsed for 5 to 10 minutes before the experiment by suspending in phosphate-Ringer solution through which a rapid stream of oxygen is passed. This precaution is found to give a greater uniformity in the amount of preformed CO_2 in the different slices. The oxygen used is freed from traces of CO_2 by passing through a long tube filled with soda-lime.

Preparation of the vessels. The vessels must be thoroughly cleaned overnight with strong chromic acid mixture and before use they must be washed very thoroughly to remove all traces of acid, and afterwards dried at 100°. 0·3 cc. of 2·5 *N* HCl is pipetted into the side-bulbs and 2 cc. of phosphate-Ringer solution are quickly added (the solution being run in from a pipette with two graduations) to the central portion of the first vessel. This vessel is immediately connected with its manometer and a rapid stream of CO_2-free oxygen is passed through and escapes around the loosely inserted stopper. The remaining vessels are similarly connected up, one at a time, and each of these in addition to the solutions contains an approximately equal piece of tissue. While the gas is rapidly passing, 0·5 cc. of barium hydroxide solution is measured into each vessel, the stopper *S* (Fig. 1) being momentarily removed for this purpose. The tip of the self-filling burette (Fig. 2) is protected by immersion in *N*/20 HCl until the moment of inserting into the vessel. If this operation is carried out with reasonable rapidity a constant and very low blank-value for the CO_2 content of the barium hydroxide solution is consistently obtained. The CO_2 content of the 0·5 cc. as determined by the control experiment should not

exceed 6 mm.^{3} and there is no difficulty in reducing the correction to this figure or less. The 2 cc. of boiled phosphate-Ringer solution contain nearly the same volume of CO_2, this value also being very consistently maintained throughout our experiments. Since the total volumes of oxygen and CO_2 measured are of the order of 400 mm.^{3}, the error introduced by slight variations in the correction for the solutions is negligible, provided that, as a check, their values are freshly determined for each experiment. Hence the use of more elaborate precautions in order to reduce this correction further is in general unnecessary, and would much reduce the simplicity of the technique.

The stoppers of the vessels are now pushed home, and the gas-stream is at the same moment diverted by turning the upper stop-cocks of the manometers.

The manometers and vessels are now attached to the shaking-apparatus by means of which the vessels are moved backwards and forwards in the thermostat [Warburg, 1926]. It is important that the vessels II and III, containing the tissue, should be put into the bath together so that the respiration in both follows the same course. When the vessels are shaken the barium hydroxide solution moves freely in the annular trough without any tendency to splash over into the central portion. This point is important, as the shaking must be sufficiently vigorous (excursion 5 cm.; 100 oscillations to the minute), in order rapidly to establish equilibrium of absorption of gas. Owing to the large surface presented by the barium hydroxide and the rapid movement, which entirely prevents the formation of any superficial crust of carbonate, the absorption of CO_2 is extremely efficient and control experiments have shown that it is superior to that of the usual conical vessels containing potash as absorbent.

Ten minutes after inserting the vessels into the bath the first reading of the pressure in all vessels is taken. This period is sufficient for complete equilibrium and should not be prolonged. After taking this reading, vessel II is at once removed from the bath and the acid contained in the side-bulb is thoroughly mixed with the contents by repeated tipping. The vessel is then replaced in the bath. After a further 15 minutes another reading is taken, and from the observed small increase of pressure the total preformed CO_2 in solutions and tissue is calculated. Readings of pressure in vessel III are taken at intervals of 15 or 30 minutes throughout the experiment and so the oxygen consumption is followed. After a definite time, measured from the tipping of vessel II, the acid is tipped into vessel III immediately after a reading has been taken. The large positive pressure set up is used for the calculation of the total CO_2 in vessel III at the end of the experiment.

As a check that all the bound CO_2 has been liberated the mixing of the contents of both vessels is repeated. There should be no further increase of pressure as a result of this second mixing.

At a convenient time during the above manipulations, the acid is tipped into vessel I, containing the solutions without the tissue. The slight positive

pressure set up (a few mm. only) gives the correction for the CO_2 content of the solutions.

It is important that the amount of tissue used and the duration of the experiment should be so chosen that nearly the whole range of the manometer capillary (300 mm.) is used, if the greatest accuracy is to be obtained. With most tissues a period of 2–3 hours is sufficient.

At the end of the experiment the weights of the pieces of tissue are determined by removing from the vessels, thoroughly rinsing in a large volume of water, and drying to constant weight at 110°.

Calculation. From the above readings of pressure the following quantities are directly determined by simple multiplication by the vessel constants (see below). The actual exchange is determined from vessel III:

(*a*) total oxygen consumption;

(*b*) total CO_2 present at the end of the experiment;

readings (*b*) have to be corrected for the following:

(*c*) from vessel II the total CO_2 present at the beginning of the experiment;

(*d*) from vessel I, the CO_2 contained in the solutions used.

By subtracting (*d*) from (*c*) the amount of CO_2 originally present in the tissue, together with that formed by respiration during the initial period of 10 minutes, is given. The weights of the tissues being known, this correction can at once be applied to (*b*), its amount being directly proportional to the weights of the tissues. It is advisable that this correction should not exceed about 10 % of the whole volume of CO_2 in vessel III—this is always the case if the experimental period and weights of tissues are suitably selected.

The corrected value of (*b*) is the respiratory CO_2 during the period of the experiment. This value divided by (*a*) gives at once the respiratory quotient. The method of calculation for a typical experiment is given in detail in the protocol at the end of the paper.

Notes.

Vessel constants. The actual volume of gas given off or consumed (mm.^{3}) is calculated by multiplication of the pressure change observed (mm. Brodie fluid) by a constant whose value depends on the volume of the vessel and the nature of the gas. The calculation of the vessel constant (k) from the volumes of the liquid and gas (V_F and V_G mm.^{3}) at a temperature $T°$ (absolute) is [Warburg, 1926]

$$k = \frac{V_a \frac{273}{T} + V_F . \alpha}{10{,}000},$$

where α is the Bunsen solubility coefficient of the gas given off or consumed.

The solubility of oxygen, which is in any case small, may be considered as being the same in Ringer solution as in the solutions used in the present method. The same is not true of the CO_2, the solubility of which is diminished by the

acidity and salt concentration of the solution obtained after mixing with acid. Since no value was available in the literature, the solubility was determined for this solution by the "first saturation method" of Austin *et al.* [1922], using the manometric Van Slyke apparatus for the estimation. The value found ($\alpha_{CO_2} = 0{\cdot}517$ at 38°: mean of four determinations) is inserted in the above equation for calculation of the vessel constants.

Accuracy. As both oxygen and carbon dioxide are determined manometrically on the same piece of tissue, the error in the actual readings (± 1 mm.) is negligible when the pressure is of the order of 250 mm. of Brodie's fluid. The error is, therefore, determined by the error in the corrections for preformed CO_2 due to tissue and solutions. The error due to variation in the CO_2 content of the solutions themselves has already been discussed. Provided the stated precautions in pipetting are observed, this error does not exceed 1 %, as we have satisfied ourselves by a large number of duplicate estimations extending over several months. In practice, therefore, the accuracy depends on the agreement between the preformed CO_2 per unit weight in the duplicate pieces of tissue (vessels II and III). The process of oxygenation of the tissue before the experiment tends to make this correction more uniform, but it is evident that the CO_2 present in the tissue and that formed by respiration in the first 10 minutes of temperature equalisation cannot be controlled as accurately as can the amount of CO_2 present in the solutions. On account of the removal of the more serious errors of previous methods, this correction becomes the determining factor in the accuracy of the present technique. However, it must be remembered that this quantity is itself a correction term the magnitude of which should not exceed about 10 % of the total CO_2. Consequently, if the error in the correction is as high as 20 %, the final error introduced will be only 2 %.

From the above considerations, the greatest error to be expected in the value of R.Q. is 0·02. A better agreement has been observed in a large number of experiments on the same tissue. These observations are included in a forthcoming paper (Part II). It is customary, in the literature of the methods discussed in the introduction to the present paper, to find the R.Q. expressed to the third decimal place. The above considerations show that such a procedure is entirely unjustified by any of these methods.

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Protocol.

Rat-brain cortex. Adult female rat fed 24 hours before experiment.

Solution. Ringer containing 0·2 % glucose and 11 m.-mols per litre phosphate-buffer p_H 7·47. Slices of tissue rinsed for 5 mins. in oxygenated solution. Duration of experiment: 3 hrs. Temp. 38°.

Vessel no.	I	II	III	IV	V	VI
Total vol. (cc.)	21·25	22·02	20·27	22·70	20·07	22·00
Side-bulb			0·3 cc. of 2·5 *N* HCl in all			
Main part			2 cc. of phosphate-Ringer in all			
Tissue	–	+	+	+	+	+
Annular trough			0·5 cc. $Ba(OH)_2$ solution in all			

Readings. After 10 mins. in bath, readings of all manometers taken.

Acid tipped in ...	Tip	Tip	No	No	Tip	No
Pressure changes at 30′ intervals corrected for thermobarometer (mm. Brodie fluid)	+ 6	+ 14·0 Tip again	– 34·0	– 25·0	+ 14·5 Tip again	– 44·0
	—	+ 0·5	– 36·5	– 24·0	—	– 42·0
	—	—	– 36·5	– 25·0	—	– 41·0
	—	—	– 34·0	– 23·5	—	– 42·5
	—	—	– 33·5	– 22·5	—	– 41·5
	—	—	– 35·0	– 24·0	—	– 43·5
	—	—	Tip	Tip	—	Tip
	—	—	+ 206·5	+ 145·5	—	+ 248·5
	—	—	Tip again		—	
	—	—	—	—	—	+ 0·5
Dry weights of tissue (mg.)	—	11·79	8·01	6·16	7·19	12·54
Area of slices (mm.²)	—	130	130	130	90	90
Thickness (mm.)	—	0·45	0·3	0·25	0·4	0·65*
Calculation:						
h_{O_2}	—	—	– 209·5	– 144·0	—	– 234·5
k_{O_2}	—	—	1·54	1·754	—	1·693
Total oxygen consumed (mm.³)	—	—	**323·0**	**253·0**	—	**431·0**
h_{CO_2}	+ 6·0	+ 14·5	+ 206·5	+ 145·5	+ 14·5	+ 249·0
k_{CO_2}	1·674	1·832	1·678	1·892	1·66	1·831
Total CO_2 (mm.³)	10·6	26·6	346·5	275·5	24·1	456·0
Correction for solutions	—	10·6	10·6	10·6	10·6	10·6
Preformed CO_2 in tissue (mm.³)	—	16 or 1·36 per mg.	—	—	13·5 or 1·88 per mg.	—
Correction for tissue (mean of II and V multiplied by weight)	—	—	13·0	10·0	—	20·4
Total CO_2 produced in mm.³	—	—	**322·9**	**254·9**	—	**425·0**
R.Q.	—	—	1·00	1·01	—	0·99
Q_{O_2} (oxygen: mm.³ respired per hr. per mg. dry weight)	—	—	– 13·4	– 13·7	—	– 11·4

* Although this piece of tissue was too thick, the value of R.Q. is unaffected.

CI. OXIDATIONS IN CENTRAL AND PERIPHERAL NERVOUS TISSUE.

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(*Received June 14th, 1930.*)

Introduction.

The experiments described in this paper were undertaken in the hope of throwing some light on the question of the oxidation mechanisms of nerve and of brain tissue. The data at present available show that the rate of oxygen consumption by the two tissues is vastly different. There can be no doubt that the brain as a whole uses oxygen at a very much greater rate than does peripheral nerve. The literature bearing on this point has been briefly discussed in previous communications [Holmes and Gerard, 1929; Holmes, 1929, 1] and will not again be referred to here. How far these data can be accepted in a strictly quantitative sense is, of course, open to question. All the experiments on the intact brain have necessarily involved the use of anaesthesia at some stage in the proceedings, while there is no means of judging how far isolated portions of brain may be expected to behave as they would *in situ*. In the experiments on nerve, the tissue has always been isolated. Still, isolated nerves can be proved to be functionally active, and it seems reasonable to assume that the oxygen which they use can be taken as a fair measure of that demanded by them while *in situ* in the living animal. On the whole, therefore, brain is likely to be more adversely affected by experimental conditions than is nerve, and probably experiments have under-rated, rather than over-rated, the true difference between the tissues.

In the present experiments, isolated rabbit or guinea-pig nerves have been employed, and their oxygen consumption has been determined by the use of a Barcroft's differential manometer. The brain tissue was that of cats, rabbits, and mice. Sometimes chopped whole brain was used; sometimes slices of cortex were employed; the oxygen uptake was observed in the same way as was that of the nerves.

1. Oxygen uptake of untreated tissue.

In the first place, the oxygen uptake of cortex slices, chopped whole brain (mixed grey and white matter), pure white matter from the spinal cord and peripheral nerve, was compared (Fig. 1). In these, and in subsequent experiments mentioned in this paper, the tissue was put up in a fluid having the

following composition: Ringer's solution 80 parts, $M/2$ acid potassium phosphate 20 parts, NaOH, sufficient to bring the p_H to 7·6.

It will be seen that the most rapid uptake is that of the cortex (it has been found that it matters little whether it is chopped or sliced) and that the uptake of whole, chopped brain is less. In the case of white matter from the spinal cord (obtained by exposing the cord and cutting off strips of the posterior columns with sharp scissors) affairs are very different. While rabbit cortex takes up about 1200 mm.^{3} of O_2 per g. of tissue per hour, white matter takes up only from 200 to 300 mm.^{3}, an amount not very much greater than that used by peripheral nerve. This is hardly a matter for surprise, since one has no reason, at present, to suppose that the conducting elements inside the

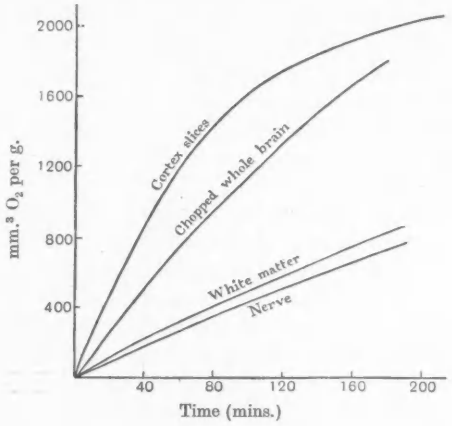

Fig. 1. Rabbit.

central nervous system differ in any essential way (except in power of regeneration) from those which carry impulses to and from the periphery. With regard to the uptake of oxygen by cortex, it must be remembered that the brain tissue was bathed only in Ringer's solution. If 0·25 % glucose is added to the fluid, the uptake in 3 hours is about doubled. In life, the cells are bathed in a fluid containing glucose, and in all probability therefore, from this cause alone, their uptake *in vivo* is much greater than these experiments indicate.

The results obtained with grey matter agree on the whole with those of Loebel [1925] (allowing for the fact that he used rat-cortex, and worked at 40°), while those for nerve tally well with Gerard's results [Holmes and Gerard, 1929] and with those of Sherif [1929]. It has been found that there is a very marked difference in the rate of oxygen consumption of brain tissue of different species, in the sense that smaller animals give tissue with a larger uptake. Loebel's values for rat-brain are rather higher than those here reported for rabbit (even allowing for the fact that he worked at a higher

temperature). Experiment shows, on the other hand, that chopped mouse-brain (whole) takes up oxygen more quickly even than rat-cortex (*e.g.* over 1600 mm.^{3} per hour). Dixon and Elliott [1929] on the other hand, report much lower values for ox-brain.

2. Oxidation mechanisms.

Attempts to investigate some of the oxidation mechanisms involved have naturally followed the lines laid down by the work of Keilin [1929][1].

A series of preliminary experiments with "Nadi" reagent left no doubt that grey matter gave a much more active indophenol oxidase reaction than did either white matter or peripheral nerve. Vernon [1911, 1912] had previously investigated the indophenol oxidase activity of various tissues, among them brain. He made his experiments quantitative by comparing the intensity of colour formation by various tissues after a given time. He observed that the intensity of the reaction varied inversely with the size of the animal. He does not seem to have employed nerve, nor to have differentiated in the brain between grey matter and white. In all cases he found that the most active reaction was given by heart muscle, and that cerebral cortex occupied a high place on the list.

Work on quantitative lines was undertaken in the present circumstances as follows. The oxygen uptake of the washed tissue, ground and suspended in buffered Ringer's solution, to which *p*-phenylenediamine was added, was observed in a Barcroft's apparatus. Without added *p*-phenylenediamine, the O_2 consumption of the suspensions was almost nil. Rabbit, mouse, ox and cat tissues were used at different times, and all gave similar results.

Cat tissues were the most convenient, since from these animals fresh tissue can be obtained in adequate amounts; they have been used to construct the curves in Fig. 2. Keilin's [1929] instructions were followed in preparing the tissue emulsions, the volumes of fluid being proportionately reduced to suit the smaller amounts of tissue. In each case, to provide a basis for comparison, 1 cc. of tissue suspension was pipetted into the cup of the Barcroft's apparatus, and a similar amount, measured with the same pipette, was introduced into a crucible, and dried at 100°. The crucible was weighed, ignited, and re-weighed, the difference giving the amount of organic matter present. The results are expressed as mm.^{3} of oxygen per 100 mg. of organic matter.

This experiment leaves no doubt that the indophenol oxidase of grey matter is much more active than that of white matter, or of peripheral nerve. The ratio of the total uptakes in this particular case is grey: white: nerve = 26·2: 4·3: 1. The difference is very much greater than that shown by the total oxygen uptakes of the three tissues, though the latter is obviously in the same direction. Since, according to Keilin, the function of indophenol oxidase is to oxidise cytochrome reduced by the tissue dehydrases, it seemed possible that the distribution of the pigment in the three tissues might follow similar lines. To

[1] See also review by Dixon [1929].

investigate this, a rabbit was anaesthetised with ether, the chest opened, and cannulae tied into the aorta and right ventricle. The pulmonary artery was clamped, and the animal perfused through the aorta with Ringer's solution kept in a reservoir at 37°, the fluid escaping through the cannula in the right ventricle. (The purpose of the perfusion is, of course, to remove haemoglobin.) When about 4 litres of fluid had been run through, the perfusion was stopped, and the brain, portions of white matter from the spinal cord (posterior and latéral columns), and the sciatic nerves were removed. The three tissues were examined for cytochrome by means of a microspectroscope by Mr R. Hill. He reported that, in the cortex, there was about half as much cytochrome as in yeast; that is, much more than in skeletal muscle, but less than in heart

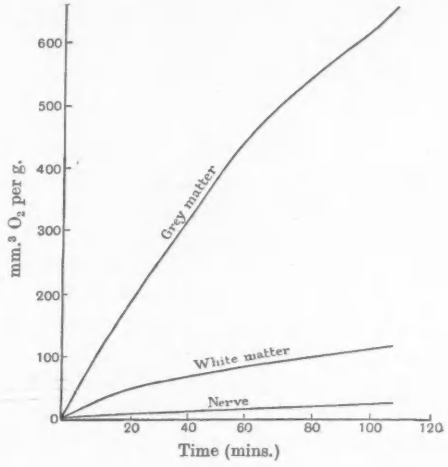

Fig. 2. Cat.

muscle. White matter from the cord contained $\frac{1}{4}$ to $\frac{1}{5}$ as much as cortex; he could not state with certainty that any of the pigment was present in the nerves. The comparison is necessarily rough, and is based on measurement of the thickness of the slices of tissue which give absorption spectra of equal intensity. It is interesting that the amounts of cytochrome in cortex and white matter are approximately in the same ratios as the intensity of the indophenol oxidase activity—about 4 to 1 in the one case and 6 to 1 in the other.

Since nerve is relatively so deficient in indophenol oxidase and cytochrome, it would be of interest to see if this fact could be correlated with its incapacity to oxidise lactic acid, a feature of its metabolism which has been previously reported [Holmes and Gerard, 1929]. Thunberg states that lactic acid will donate hydrogen to methylene blue in the presence of nerve under anaerobic conditions; it seemed, therefore, possible that methylene blue might act as a carrier of hydrogen, in the presence of oxygen, and enable lactic acid to

be oxidised, thus replacing the absent oxidase system. Nerve was therefore put up in a Barcroft's apparatus with 0·5 % lithium lactate, in buffered Ringer-phosphate (p_H 7·6) containing methylene blue $M/2500$. Some experiments showed no increase in oxygen uptake in the presence of methylene blue and lactate, others an increase so slight that it cannot be accepted as significant.

3. Dehydrase systems.

The fact that nerve tissue will reduce methylene blue in the absence of oxygen [Thunberg, 1923; Sherif, 1929] shows that it possesses dehydrase systems, and Thunberg reports that certain substances—glutamic acid, ketoglutaric acid, succinic, fumaric, and lactic acids—are capable of acting as hydrogen donators to methylene blue. Sherif finds that neither the presence of glucose nor of galactose effects any decrease in the reduction time.

Herter [1905] showed that methylene blue was reduced by brain tissue *in vivo*, and was re-oxidised again if the brain was exposed to the air after the death of the animal. Szent-Gyorgyi [1924] found that succinic acid acted as a hydrogen donator to brain tissue *in vitro*. He was able to show that, in the case of brain (unlike that of other tissues), the rate of oxidation of succinic acid was not limited by the rate of activation of oxygen. It has already been remarked that, in the present experiments, methylene blue was found to have no effect on the oxygen uptake of nerve. Table I shows some results obtained during the course of the present work with grey and white matter of ox-brain.

Table I.

	Reduction times in mins.	
	Grey matter	White matter
Control	13½	70
$M/50$ Lithium lactate	7	24
$M/100$ Glucose	6	48
$M/100$ Galactose	10	70

It is quite evident, from these figures, that the dehydrase mechanisms of grey matter, like the indophenol oxidase, are far more active than are those of white matter.

Since grey matter consumes oxygen so readily, and since its oxidation mechanisms seem to act so much more intensely than those of white matter or nerve, it is of interest to see what information is available as to the substrates which the three tissues are able to use.

It is well known that cortex, or whole brain, causes the rapid disappearance of lactic acid, provided oxygen is available [Warburg *et al.*, 1924; Holmes and Holmes, 1925]. It is also known that the tissue can convert glucose to lactic acid with great rapidity. From Table I it will be seen that the reduction time of methylene blue by ox-cortex is about equally rapid in the presence of glucose, as it is in that of lactic acid. To decide whether glucose was oxidised as such, or was first converted to lactic acid, advantage was taken of the fact that

fluoride would prevent the formation of lactic acid by brain tissue [see Ashford and Holmes, 1929].

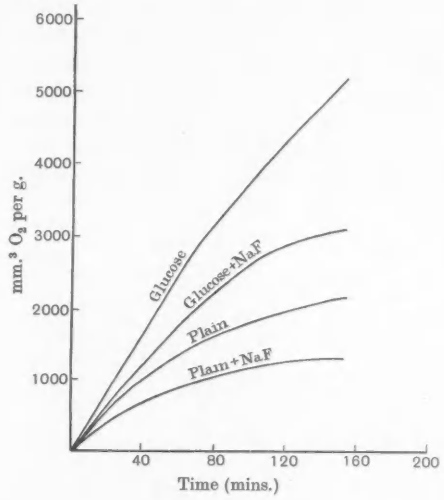

Fig. 3. Mouse.

The present experiment was arranged as follows. Equal amounts of chopped brain tissue (rabbit and mouse) were put up in each of four Barcroft's apparatus[1]. The cups of the first contained plain Ringer's solution, buffered with phosphate to p_H 7·6. The second contained a similar solution plus 0·25 % glucose, the third, as well as the glucose, 0·01 *M* sodium fluoride, and the fourth 0·01 *M* sodium fluoride, but no glucose. The curves of oxygen uptake are given

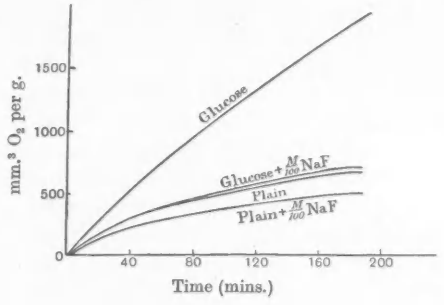

Fig. 3*a*. Washed mouse brain.

in Fig. 3. It will be seen that the extra oxygen uptake due to the glucose is very greatly inhibited by the fluoride. There is also some inhibition of uptake

[1] I have to thank Mr C. A. Ashford for assistance with these experiments.

by the fluoride in the case of the tissue to which no glucose was added. This presumably indicates the inhibition of lactic acid formation from some precursor already present in the tissue. To test this point, the experiment was repeated with washed tissue. Washing very much decreases the oxygen uptake of chopped brain, and the uptake is restored by adding glucose. Fig. 3 *a* shows that this restoration is largely prevented by 0·01 *M* NaF. At the same time, fluoride still has some effect on the reduced uptake which goes on in the absence of glucose, suggesting that perhaps here it exerts an effect on the metabolism of other substances besides lactic acid—a very probable happening, in view of the findings of other workers.

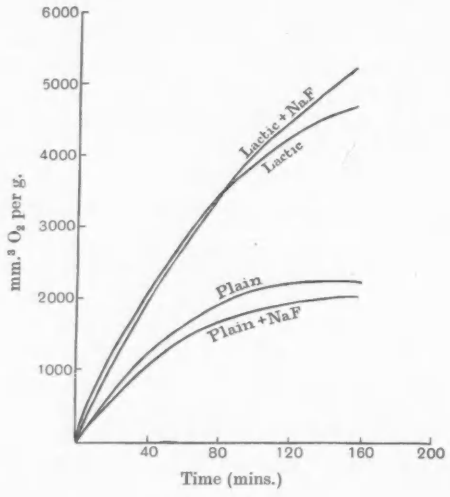

Fig. 4. Mouse.

It may be remarked that from previous experience [Ashford and Holmes, 1929] a complete inhibition of lactic acid formation by 0·01 *M* NaF was not to be anticipated; the figures previously reported indicated an inhibition of about 80 %.

If now, instead of glucose, lithium lactate be used in the above experiment, there is no inhibition of oxygen consumption: the "lactate" and "lactate and NaF" curves are almost identical (Fig. 4). It seems safe, therefore, to conclude that glucose must be converted into lactic acid before it can be oxidised by the grey matter.

As has previously been reported [Sherif and Holmes, 1930] the only effect of the addition of glucose to peripheral nerve is to prolong the period during which a nerve is able to take up oxygen at an approximately linear rate. Even this effect is usually only discernible during the course of an experiment deliberately prolonged for many hours. The same appears to be true of white

matter from the cord, though the point has not yet been very fully investigated; it can, however, be said with certainty that in the case of such white matter there is no marked increase in oxygen uptake in the presence of glucose, such as is invariably obtained with grey matter in similar circumstances. It has been shown [Holmes and Gerard, 1929] that there is no disappearance of lactic acid from peripheral nerve when the tissue is kept in oxygen. It is, therefore, to be expected on all these grounds that fluoride has no influence on the oxygen uptake of nerve. Fig. 5 shows that this expectation is realised.

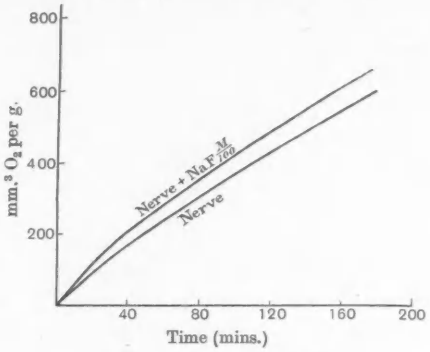

Fig. 5. Guinea-pig nerve.

4. Oxidation of lactic acid by "central" white matter.

Most of the facts so far elicited seem to suggest that white matter from the central nervous system behaves very much like peripheral nerve, a finding which at least fits in with anatomical and histological conceptions. It is, therefore, disconcerting to find that white matter incubated aerobically seems to possess some power of causing the disappearance of lactic acid. Its performance in this respect is a very poor one compared with that of grey matter; it seems, however, that it must be accepted as real.

The results of a number of experiments are given in Table II. The amounts of tissue available when rabbits or cats were employed was small, and the experimental error correspondingly large (in the neighbourhood of 10 %). The final experiment, however, in which ox-brain was used and ample tissue was available, seems to put the matter beyond doubt.

The white matter, in most of these experiments, was placed in a cup of a Barcroft's apparatus and shaken in a thermostat at 37° for 3 hours, the oxygen uptake being measured at the same time. The tissue is much more friable than is peripheral nerve, and at the end of the experiment is, for the most part, broken up; nerve, of course, in similar circumstances, remains intact. An experiment with finely chopped nerve did not, however, show any fall in lactic acid, so that apparently the phenomenon is not dependent upon the mechanical disintegration of the tissue.

Table II.

	Lactic acid: mg. per 100 g. fresh tissue			O_2 absorbed (mm.³)	Duration of exp. (hours)	Temp. 0°	Tissue
	Initial	Final	Change				
1*	108	88	− 20	—	2½	37	Cat's cord
2†	102	40	− 62	885	3	37	Rabbit's cord
3	93	109	+ 16	781	3	37	,,
4	107	115	+ 8	845	3	37	,,
5	119	81	− 38	751	3	37	,,
6	134	102	− 32	830	3	37	,,
7	83	84	− 1	566	3	37	,,
8	118	110	− 8	794	3	37	,,
9	116	109	− 7	575	3	37	Cat's cord
10	102	72	− 30	—	3	37	Rabbit's cord
11‡	153 (*a*)	120	− 33	—	3	37	Ox-brain
	154 (*b*)	121	− 33	—	3	37	,,
Average	116	96	− 20	753	—	—	

* Tissue kept in a tube in O_2. All remainder shaken in air.
† Extraction with trichloroacetic acid used for estimation. All remainder worked up by technique described by Holmes and Gerard.
‡ "*a*" and "*b*" duplicate samples, each of 3 g.

5. Effect of washing nerve and brain tissue.

Whatever the materials may be which are responsible for the oxygen consumption of nerve, they are not easily washed away. There is very little difference between the oxygen uptake of nerve that has been soaked in Ringer's solution for 4 hours and one kept in a moist chamber for the same period (Fig. 6). The observations of Meyerhof and Schmidt [1929] on the R.Q. of resting nerve suggest that fats are being oxidised, and this, perhaps, fits in with the foregoing observations.

In contrast to this, soaking the chopped brain tissue for 4 hours in Ringer's solution reduces the oxygen uptake enormously (Fig. 6): in contrast to nerve, therefore, either the brain depends chiefly on freely diffusible substrates, or its oxidising mechanisms are damaged by this treatment. To try to decide between these possibilities, two mice were killed, their brains chopped and sampled, and the chopped tissue soaked for 4 hours in Ringer's solution. The oxygen uptake of the washed tissue was very small, but was very much increased by the presence of 0·25 % glucose in the fluid (compare Fig. 3 *a*). In another experiment, it was found that 0·5 % lithium lactate had an even more pronounced effect. Clearly, in brain as in many other tissues, washing removes substrates, but leaves oxidising mechanisms intact.

That lactic acid is not the only substance oxidised by brain tissue is clear from a consideration of the following experiments.

(*a*) 2 g. of chopped rabbit brain were shaken in a Barcroft's apparatus[1] at 37° in air for 276 minutes. During this time, they consumed 5393 mm.³ of oxygen. Another 2 g. sample of the same lot of tissue gave an initial value for

[1] Apparatus specially constructed to deal with large amounts of tissue, to be described later in reporting other work.

lactic acid of 2·53 mg. The oxygenated sample contained at the end of the experiment 0·41 mg. 2·13 mg. had therefore disappeared.

Supposing this to have been oxidised, it would have required 1555 mm.^{3} of oxygen: 3838 mm.^{3} of oxygen are therefore "surplus" and must have been used for oxidising other substances. It may be objected that there is no proof that lactic acid was not formed and oxidised during the course of the experiment, so that, in reality more disappeared than is indicated by these figures. We have previously shown, however, that the anaerobic increase in lactic acid is small [Holmes and Holmes, 1925; Ashford and Holmes, 1929]: there is

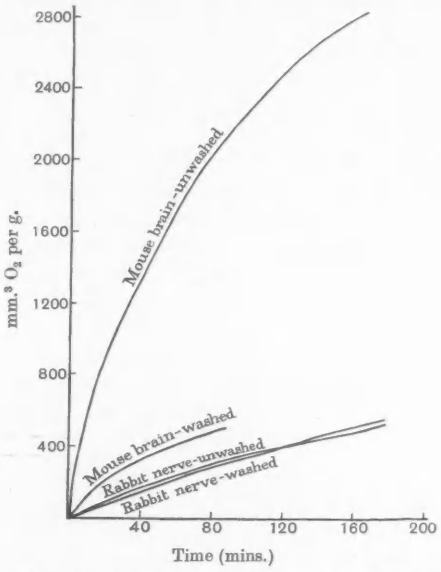

Fig. 6. Mouse brain and rabbit nerve: effect of washing.

very little precursor available in the tissue, and 2·53 mg. is nearly, if not quite, a maximum value. At the most, an extra 0·5 mg. of lactic acid might have appeared.

(*b*) Six mice were starved for 12 hours. Three of them were then given 4 units each of insulin (Burroughs Wellcome) subcutaneously. After $1\frac{3}{4}$ hours, these three all showed severe hypoglycaemic symptoms. Both groups of mice were then killed, their brains excised, and the two groups of brains separately chopped and sampled. 100 mg. of tissue were then taken from each group, and the oxygen uptake observed (Fig. 7). The remainder of the tissue was used for lactic acid determinations, which gave the following values: normals 1·148 mg. per g.; insulin 0·560 mg. per g.[1]

[1] I have to thank Mr C. A. Ashford for performing these estimations.

It is obvious that the oxygen consumed by the brains of the insulin-treated animals is less than that consumed by the normals. It is also plain that there is a very definite oxygen consumption still taking place even though there is very little lactic acid available.

It would seem, reviewing the evidence that has so far been brought forward, that we have to do with two very different types of metabolism in the brain and in the purely conducting parts of the nervous system. Such a conclusion only bears out many already well-known facts of physiology, such as the great sensitivity of the brain, and the relative insensitiveness of nerves to oxygen lack. We must assume for the present that these differences are characteristic of the nerve cell, or perhaps of the synapse on the one hand, and of the conducting elements on the other. The rate of oxygen consumption of conducting elements is of the same order, whether they be central or peripheral. Nerve cannot oxidise lactic acid, either at rest or during activity

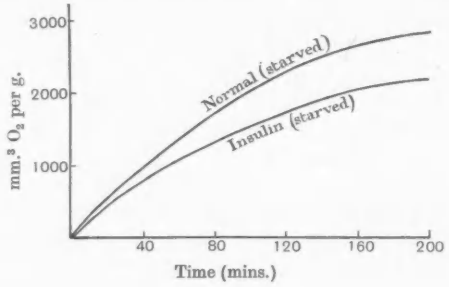

Fig. 7. Mouse.

[Holmes, Gerard and Solomons, 1930]. "Central" white matter can do so, but only to a limited extent. Carbohydrate disappears from resting nerve if oxygen is available, but, even if it is oxidised, it can account for only a part of the observed oxygen consumption [Holmes and Gerard, 1929].

In grey matter, replacing, or perhaps superimposed upon, a metabolism of this type, there appears to be another of a more intense kind. Oxygen is used far more freely, and the substance oxidised is certainly, in great part, lactic acid, though there is clear evidence that other substrates contribute a very appreciable quota.

The tissues dealt with in these experiments have been medullated nerve and nerve cells from the mammalian central nervous system. It is worth recalling that both the heat production [Hill, 1929] and the oxygen consumption [Meyerhof and Schultz, 1929] of non-medullated nerve are far greater than those of medullated nerve, and that the carbohydrate content [Holmes, 1929, 2] of the former is many times greater than that of the latter. No cytochrome could be detected by the writer in non-medullated nerve ganglion.

SUMMARY.

1. It has been shown that the various types of tissue that compose the central nervous system in mammals have different rates of oxygen consumption, and that the activity of grey matter is in this respect far greater than that of white matter, whether the latter forms part of the central system or is taken from a peripheral nerve. The term grey matter necessarily includes without discrimination both nerve cells and synaptic junctions.

2. The distributions of indophenol oxidase and of cytochrome run (roughly) parallel both to each other and to the rate of oxygen consumption of the different types of tissue.

3. The greatly increased oxygen uptake which grey matter displays in the presence of glucose is dependent upon the conversion of the glucose to lactic acid, and disappears if this conversion is prevented by fluoride.

4. Besides lactic acid, there are certainly other substrates responsible for some of the oxygen uptake by brain tissue; and as these substrates are not easily removed by washing, they are probably not carbohydrate in nature.

5. Carbohydrate is probably not responsible for any considerable part of the oxygen uptake of nerve, but this statement must carry the reservation that little or nothing is known of the part played by the carbohydrate fraction of the cerebrosides.

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CII. SELECTIVE FERMENTATION. I.
ALCOHOLIC FERMENTATION OF GLUCOSE, FRUCTOSE AND MANNOSE MIXTURES.

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The knowledge of the chemical reactions involved in alcoholic fermentation and related glycolytic processes has been augmented in recent years by numerous researches on the later stages leading to the final products, carbon dioxide and alcohol. Although the individual enzymes forming the complex catalytic system "zymase" have not been separated, nor their scope, specificity, mode of action, and other properties definitely established, future findings may be expected to conform with our general conception of the mechanism of fermentation. Comparatively little is known of the primary reactions leading from carbohydrate to structures of three carbon atoms. The observation of Harden and Young coupling the evolution of carbon dioxide with the phosphorylation of a quantitatively correlated amount of sugar is the bridge between the two stages of alcoholic fermentation. Analogies occur in the carbohydrate metabolism of animal cells.

The most striking feature of glycolysis, its high stereochemical specificity, remains to be explained. A number of investigators, Dubrunfaut [1847], and especially Bourquelot [1886] and Slator [1908], demonstrated that the "zymohexoses," glucose, fructose and mannose are fermented at about equal rates when offered singly to yeast, but from a mixture like invert sugar, glucose is preferred to fructose. Gayon and Dubourg [1890] were the first to observe that some Sauterne wine yeasts behaved differently and preferred the laevorotatory moiety of invert sugar. The selectivity of dried yeast (zymin) and yeast juice (Buchner's zymase) usually parallels that of the fresh yeast from which they are prepared, but in some cases Fernbach, Schoen and Mori [1927] observed a reversion of selectivity. Fernbach and Schiller [1924] noted that culturing in alkaline media will influence the selective power of a "glucose"-yeast in the direction of increased fructose fermentation. The laws of these phenomena and of similar ones observed in other sugar mixtures will offer a clue to the mechanism of fermentation.

The speed of fermentation is independent of the sugar concentration within wide limits, and depends only on the concentration of the yeast. As the final

[1] Isadore Hernsheim Research Fellow, 1929.

step of alcoholic fermentation, the evolution of carbon dioxide and alcohol, is common for all fermentable sugars, it is not surprising that the formation of the end-products from a mixture should be controlled by the amount of carboxylase present. Carbon dioxide is released at a slower rate from the mixture than the sum of the rates in separate fermentation; the rate of fermentation is barely greater for the mixture than that of the faster fermenting component (cf. Table II, last column). The fermentation of glucose and fructose was treated quantitatively by Willstätter and Sobotka [1922]. The selectivity of a yeast is expressed by a factor $K_{G/F} = \frac{\ln y_0 - \ln y}{\ln z_0 - \ln z}$, where y_0 and z_0 are the initial concentrations of glucose and fructose and y and z their concentrations at any given time. It was found that the average factor was $K_{G/F} = 2{\cdot}94$ for Munich Loewenbräu yeast. This factor changed to 1·89 when the yeast was cultured on a fructose medium. Hopkins [1928; cf. Ivecovich, 1930], using the same formula, found $K_{G/F}$ about 1·8 for an English top brewery yeast; this figure was unaffected by culturing on fructose.

In view of the great variations of enzymic content with type and strain, we determined the selectivity of various yeasts in the present study. We found 2·10–2·21 for a bottom brewery yeast (New York Lager), 2·46 for American baker's yeast, 2·98 for a top brewery yeast (New York Ale), and 4·63 for *Saccharomyces Marxianus*. We tried to verify the preference for fructose in two strains of Sauterne yeast which we obtained from the Versuchs-Anstalt für Brauwesen in Vienna and from the Institut für Gärungsgewerbe in Berlin. However, they showed a preference for glucose. Culturing the Vienna strain on fructose reduced $K_{G/F}$ from 4·0 to 2·6 and subsequent cultures on grape juice reached $K_{G/F} = 2{\cdot}33$, which still signifies a preference for glucose. This limited adaptation to fructose resembles the behaviour of Munich brewery yeast. The $K_{G/F}$ of the New York Lager yeast was not reduced by culturing on fructose; it is analogous in this respect to Hopkins's brewery yeast which was unchanged by fructose treatment. $K_{G/F} = 2$ seems to be the lower limit of fructose adaptation. This limit was not reached by the Berlin strain which showed $K_{G/F} = 2{\cdot}8$ to 2·9 when cultured on fructose. The yeasts which were transferred from fructose to maltose media reached $K_{G/F} = 4{\cdot}0$ and 3·6 and the Berlin yeast also reached $K_{G/F} = 3{\cdot}85$ when removed from an active fermentation of saccharose before the glucose component had disappeared.

Relation of selective fermentation to saccharase and maltase.

According to recent investigations of Weidenhagen [1930] the multiplicity of saccharase, maltase, raffinase and related enzymes can be reduced to one α-glucosidase and one β-fructosidase. As these enzymes are instrumental in the formation of fructose and glucose from higher saccharides, we surmised a correlation between these specific hydrolysing enzymes and the analogous specific zymases. However, treatment of yeast as devised by Willstätter, Lowry and Schneider [1925] to increase or decrease the saccharase content

failed to change the selectivity for glucose-fructose fermentation. This selectivity was also unaffected by the addition of large quantities of purified saccharase.

S. Marxianus contains fructosidase but no glucosidase, as it does not hydrolyse or ferment maltose; nevertheless, it has a decided preference for glucose over fructose.

Fermentation of mixtures containing mannose. The average $K_{G/M}$ was 3·8 with wide variations for the Lager yeast, although the rate of fermentation for the two aldoses was practically equal under the experimental conditions. Even fructose was preferred to mannose at a rate of $K_{F/M} = 1{\cdot}39$. The value of 2·73 for $K_{G/F}$ calculated from the two mannose factors tallies with those obtained experimentally in glucose-fructose mixtures.

Experimental.

The specific rotation of the sugars used was verified and $[\alpha]_D = -93{\cdot}6 + 0{\cdot}64\ (t^\circ - 20)$ [Laborde, 1913] was used for the temperature correction of fructose. The original brewery yeasts contained 75 % water on the average. All the other yeasts were cultured in synthetic media containing 4 % of the respective sugar besides 0·25 % KH_2PO_4, 0·25 % peptone, 0·025 % $MgSO_4$, 7 aq and 0·01 % $CaSO_4$ [Willstätter and Steibelt, 1921] and isolated after a number of 2–3 day passages by centrifugation (average moisture = 75 %).

The fermentations were carried out at room temperature. 50 cc. of a 20 % yeast suspension were mixed with 75 cc. of a 10 % sugar solution and samples withdrawn at the times given. The progress of fermentation was followed in the van Iterson-Kluyver apparatus [van der Haar, 1920] with 1 cc. of the fermentation mixture containing 60 mg. sugar. The theoretical amount of carbon dioxide from this amount of sugar with the necessary corrections for absorption, temperature and pressure is 15·6 cc. at 20° and 760 mm. The figures given in Table I under CO_2 represent volumes measured in % of the theoretical total. Fermentation in the samples was stopped by addition of a few crystals of sulphosalicylic acid and rapid centrifugation. The clarified supernatant liquid was analysed polariscopically and by the Hagedorn-Jensen method in Hanes's modification or by the Bertrand method. Corrections were applied for the yeast blank when necessary. The reduction of the sugar mixtures was interpolated from the reduction values of their constituents.

Table I gives examples for each yeast used. $K_{G/F}$ was computed by using the decimal logarithms of the sugar concentrations as given in the preceding columns in % of total sugar originally present.

A synopsis of our experiments is given in Table II. The time necessary for evolution of half the theoretical amount of carbon dioxide was noted in the Kluyver apparatus. The relative speeds of fermentation for glucose, invert sugar and fructose were referred to that of fructose = 1. These figures are recorded in the last two columns of Table II. The deviation from unity is, with very few exceptions, less than 10 %, in accordance with previous investigations.

Table I. *Determination of $K_{G/F}$ (ratio glucose fermentation/fructose fermentation) in various types of yeast.*

Yeast and temp.	Time (min.)	Sugar (actual %)	Optical rotation $l = 2$ dm.	Specific rotation	Not fermented in % of total original sugar: Total sugar	Glucose	Fructose	$K_{G/F}$	CO_2 %
1. Lager 30°	0	6·30	− 2·26°	− 17·92°	100·0	49·6	50·4	—	—
	60	5·04	− 2·37	− 23·51	80·0	36·5	43·5	2·08	—
	90	4·40	− 2·38	− 27·07	69·8	30·0	39·8	2·13	—
	120	3·74	− 2·32	− 31·01	59·4	23·9	35·5	2·08	—
								2·10	
2. Ale 21°	0	6·48	− 2·02	− 15·6	100·0	53·2	46·8	—	—
	120	5·57	− 2·31	− 20·75	86·0	42·7	43·3	2·85	—
	210	4·66	− 2·62	− 28·1	71·9	32·1	39·8	3·12	—
								2·98	
3. Baker's 20°	0	6·55	− 2·99	− 22·8	100·0	48·65	51·35	—	—
	130	3·37	− 3·04	− 45·1	51·5	17·25	34·25	2·56	30·3
	215	1·40	− 1·89	− 67·7	21·3	3·85	17·45	2·35	54·5
								2·46	
4. *S. Marx.* 21·5°	0	6·14	− 2·57	− 20·9	100·0	49·55	50·45	—	—
	150	4·58	− 3·14	− 35·25	74·6	29·5	45·1	4·65	18·6
	255	3·64	− 3·43	− 47·1	59·35	18·75	40·6	4·49	30·1
	335	2·96	− 3·49	− 58·8	48·35	11·35	37·0	4·76	39·8
								4·63	
5. Vienna Saut. on gr. juice 17·5°	0	6·31	− 2·59	− 20·5	100·0	50·5	49·5	—	—
	70	5·73	− 2·64	− 23·0	90·8	44·4	46·4	2·17	—
	115	5·20	− 2·75	− 26·45	82·4	38·3	44·1	2·40	—
	160	4·53	− 2·81	− 31·0	71·8	31·1	40·7	2·48	—
	245	4·00	− 2·70	− 33·75	63·4	26·35	37·05	2·25	—
								2·33	
6. Berlin Saut. on fructose 20°	0	5·60	− 2·62	− 23·4	100·0	48·0	52·0	—	—
	95	4·54	− 2·81	− 30·9	81·0	34·75	46·35	2·82	16·0
	145	3·60	− 2·93	− 40·7	64·3	23·3	41·0	3·04	34·0
	197	3·08	− 2·77	− 44·7	55·0	18·4	36·6	2·73	42·3
								2·90	
7. Berlin Saut. on sucrose 20°	0	5·74	− 2·60	− 22·7	100·0	48·35	51·65	—	—
	50	4·66	− 2·99	− 32·1	81·4	34·3	47·1	3·74	14·7
	85	3·50	− 2·99	− 42·75	61·1	19·9	41·2	3·93	26·3
	130	3·01	− 3·01	− 50·0	52·6	14·1	38·5	4·19	40·4
	190	1·99	− 2·46	− 62·0	34·7	6·05	28·65	3·53	53·8
								3·85	
8 *a*. Lager +saccharase 21°	0	6·46	− 2·44	− 18·9	100·0	50·9	49·1	—	—
	90	3·78	− 2·56	− 33·85	58·5	23·8	34·7	2·19	32·7
	135	2·28	− 2·32	− 50·9	35·3	10·2	25·1	2·41	50·0
								2·30	
8 *b*. Lager (control) 21°	0	6·40	− 2·49	− 19·45	100·0	50·55	49·45	—	—
	90	3·79	− 2·62	− 34·55	59·2	23·8	35·4	2·25	33·3
	135	2·26	− 2·22	− 49·15	35·3	10·6	24·7	2·25	51·2
								2·25	
							Mannose	$K_{G/M}$	
9. Lager 26°	0	6·40	+ 4·08	+ 31·85	100·0	46·0	54·0	—	—
	60	4·70	+ 2·61	+ 27·75	73·5	25·9	47·6	4·55	—
	90	3·70	+ 1·87	+ 25·2	57·8	16·55	41·25	3·80	—
	120	2·90	+ 1·37	+ 23·85	45·3	11·35	33·95	3·02	—
								3·8	
						Fructose	Mannose	$K_{F/M}$	
10. Lager 26°	0	6·38	− 4·70	− 36·8	100·0	49·1	50·9	—	—
	60	4·40	− 3·00	− 34·2	68·9	32·1	36·8	1·30	—
	90	2·44	− 2·10	− 30·8	53·5	23·2	30·3	1·44	—
	120	2·54	− 1·44	− 28·4	39·8	16·35	23·45	1·42	—
								1·39	

Table II. *Ratio of glucose fermentation/fructose fermentation in separate and mixed solutions.*

Yeast	Exp.	Remarks	$K_{G/F}$ (for mixture)	Ratio for rate of separate ferm. gluc./fruct.	Ratio invert sugar/ fructose
Lager	No. 1.	Orig. (12. vii. 1929)	2·10	1·06	1·03
		Orig. (22. xi. 1929)	2·18	—	—
		Orig. (4. xii. 1929)	2·21	0·91	1·07
		Grown on fructose	2·50	0·98	1·11
	No. 8 *a*.	Saccharase added	2·30	0·98	1·32
	No. 8 *b*.	Control	2·25	1·02	1·20
		Enriched in saccharase	2·24	0·77	1·00
		Poor in saccharase (acid treatment)	2·13	1·00	—
Vienna Sauterne		Grown on maltose	3·98	0·98	0·98
		Grown on fructose I	3·12	1·06	1·00
		Grown on fructose II	2·60	1·04	1·01
		Grown on glucose	2·89	0·95	0·95
	No. 5.	Grown on grape juice	2·33	1·15	—
		Recultured on maltose	3·97	1·00	1·03
Berlin Sauterne		Grown on fructose	2·80	0·87	1·16
		Grown on maltose	3·62	1·00	0·97
		Grown on saccharose	2·80	0·89	0·98
	No. 7.	Grown on saccharose (interrupted)	3·85	0·98	1·19
	No. 6.	Grown on fructose	2·90	0·99	1·01
Ale	No. 2.	Orig. (5. xii. 1929)	2·98	1·21	0·85
Baker's	No. 3.	Orig. (10. xii. 1929)	2·46	1·00	0·99
S. Marxianus	No. 4.	Grown on saccharose	4·63	0·98	1·40

The saccharase determination of the Lager yeast yielded a saccharase value [Schneider, 1929] of 0·0044. By the stimulating treatment of Willstätter, Lowry and Schneider [1925] this value was more than doubled; by acid treatment it was reduced to 0·0017. The $K_{G/F}$ of these yeasts did not differ from the values for the original yeasts.

In Exp. 8, 166 mg. purified "Invertase scales" of a saccharase value 0·325, comprising the saccharase content of more than 10 g. yeast, were added to 100 cc. of a Lager yeast fermentation. No change of $K_{G/F}$ was noted.

Summary.

1. The factor $K_{G/F}$ for several types of yeast was determined. All types preferred glucose to fructose at a rate of 2 : 1 or higher.

2. This factor is subject to changes depending on the culture medium used.

3. No correlation was found between selective fermentation and saccharase or maltase content.

4. The selective fermentation of mixtures of mannose plus glucose and mannose plus fructose was studied.

We wish to thank Mr Leo Wallerstein for samples of yeast and of concentrated invertase.

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CIII. THE HEAT-STABILITY OF THE (ANTI-DERMATITIS, "ANTI-PELLAGRA") WATER-SOLUBLE VITAMIN B_2. II.

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(*Received June 19th, 1930.*)

The following experiments form a continuation of those recently published by Chick and Roscoe [1930] which indicated that vitamin B_2 in yeast or yeast extracts, though relatively stable to temperatures at 100–120° in acid solution, was sensitive to these temperatures if the reaction were alkaline. Thus, at p_H 5·0–3·0 there was a 50 % loss in vitamin B_2 content after heating for 4–5 hours at 123°; at p_H 8·3–10·0 a loss of between 75 and 100 %. When heated for 2 hours at 90–100° in faintly acid solution no loss in potency was detected; at p_H 8·3, on the other hand, about one-half was lost and even when maintained for 10 days at room temperature (summer) in a weakly alkaline solution there was a loss of about one-third of the original strength. Vitamin B_2 was estimated by the method previously described [Chick and Roscoe, 1928].

These conclusions are not in accord with those of Reader [1929, 1930], who found vitamin B_2 able to withstand heating for 1 hour at 120° at p_H 9·0, or of Narayanan and Drummond [1930], who state that no appreciable destruction of this vitamin takes place when yeast products are heated for 1–3 hours at 110–120° in a solution containing 10–15 % barium hydroxide.

This divergence is of more than usual interest, for the stability of vitamin B_2 to heat and alkali is important in the chain of arguments indicating the existence of Reader's third water-soluble B vitamin. This new factor is stated to differ from vitamin B_2 in being sensitive to heat and to alkali.

Criterion used and methods employed in estimation of vitamin B_2.

In the experiments of Reader and of Narayanan and Drummond, as well as in our own, the criterion used in the detection and assay of vitamin B_2 was the power to promote or restore growth in young rats receiving diets complete in every other respect. It is obvious that a more satisfactory criterion than growth, because more specific, would be the cure of the dermatitis (? rat-pellagra) which develops in young rats after several weeks of vitamin B_2 deficiency. Such experiments are by nature unsuited for quantitative work owing to the irregularity of these symptoms as regards their nature, time of onset and degree of severity. Nevertheless, it was felt that they might provide

useful confirmation of previous work [see Chick and Roscoe, 1930, p. 111] and in this hope the following experiments have been carried out. The results have confirmed our previous findings in demonstrating that the curative property for ratdermatitis, present in yeast extract equally with the power to restore growth of rats on diets lacking only vitamin B_2, is sensitive to high temperatures in alkaline solution.

Technique of the curative tests.

The increase in body weight of young growing rats is extremely sensitive to deprivation of vitamin B_2. When these animals are placed on diets from which this vitamin is absent, increase in weight usually ceases within a few days and it is only in exceptional circumstances (as for example in the condition known as refection [Fridericia *et al.*, 1927; Roscoe, 1927]) that an approach to even subnormal growth is manifested. The onset of the characteristic dermatitis is, however, more irregular. Sometimes it is marked after 6–8 weeks of deprivation; on the other hand, it may take 20 or more weeks to develop. The distribution, character and severity of the skin affection also vary greatly [Chick and Roscoe, 1928]. The curative tests described below were carried out on a collection of "negative" control rats belonging to different litters which had been maintained for various periods on the standard "$-B_2$" diet for the express purpose of studying these skin lesions. As will be seen from the details set out in the accompanying table, these varied considerably, but in selecting animals for the tests care was taken that, as far as was possible, the doses of the materials compared were administered to animals whose condition was similar.

The symptoms usually present when treatment began were the following. Sunken eyes with blood-stained secretion and inflamed eyelids, inflamed skin, bare of fur, surrounding the eyes giving a characteristic "spectacled" appearance. The fur was thin and poor, especially over the head, neck and chest; in severe cases the bald patches often developed into raw areas spreading round the axilla and down the forearm. The wrists of the forepaws were almost invariably stained with the bloody discharges from eyes and nostrils, as the result of incessant rubbing. The fur of the lower part of the abdomen was also stained reddish brown with what appeared to be blood from the urine. These stains, however, unlike those on the wrists, have repeatedly failed to give the characteristic tests for blood or blood-pigments.

The stains in both places are alike the first symptoms to disappear, often within a day or two after treatment with an adequate curative dose. Next in order, the eyes usually recover their normal prominent position and the skin begins to assume a healthy texture and appearance. Lastly, new fur makes its appearance, 2–3 weeks being needed before the coat may be described as normal.

A set of skin appearances differing somewhat from the above was frequently encountered in our earlier work, but now rarely occurs [Chick and Roscoe, 1928, p. 795]. These include (1) inflammation of the tips of the ears, which

become cheesy in texture and, during the cure, shed their dead epidermis in flakes, (2) an oedematous inflammation of the digits of the paws, which become bright red in colour. On one occasion these symptoms made their appearance while cure of a rat (564 ♀) with general dermatitis was being attempted with an inadequate dose of material containing vitamin B_2, suggesting the possibility that a small, though inadequate, amount of vitamin B_2 in the diet was necessary for demonstration of these more florid skin symptoms of vitamin B_2 deficiency [see also Sherman and Sandels, 1929].

It is worth noting that these particular symptoms of vitamin B_2 deficiency have been less frequent since the use of more highly purified caseinogen in the basal diet, although the failure to grow and the development of a generalised dermatitis have been more constant. The suggestion that these florid symptoms are induced by a less complete deprivation of vitamin B_2 is confirmed by the fact that, on one occasion, a rat (456 ♀) exhibiting these symptoms, but otherwise in good condition, was slowly cured when treated with a yeast extract which had been autoclaved at 120° for 4 hours in alkaline solution, this being the only instance where material so treated has, in our experience, exhibited curative properties for the dermatitis caused by vitamin B_2 deficiency.

An analogous instance is probably that of rats receiving diets almost, but not entirely, deprived of the antineuritic vitamin B_1. In the course of several weeks such rats will often develop the characteristic paralysis due to vitamin B_1 deficiency, whereas those suffering complete deprivation usually die in 3–4 weeks from collapse without manifestation of any nervous affection [Hofmeister, 1922, 1, 2].

Vitamin B_2 material used in the present tests.

A 0·01 % acetic acid extract was prepared from washed brewer's yeast, sample XXIV, similar to that made from yeast XII, used in the experiments of Series B (Table II) described in the previous paper [Chick and Roscoe, 1930]. One portion (*a*) of the extract was acidified to p_H 2·5, and a second portion (*b*) made alkaline by addition of sodium hydroxide to p_H 10·3, the hydrogen ion determinations being made with a hydrogen electrode. After heating for 4 hours at 119° the p_H of (*b*) (which had fallen to 9·6) was adjusted to about 3·0 for storage. That of (*a*) remained at 2·5. Both solutions were adjusted to their original volume and in the following tests of the curative power of the two solutions all doses are reckoned as the equivalents of the original yeast (dry weight).

No tests were made with the unheated material, as it was felt that no accurate quantitative results could be expected from such curative tests and that it would be sufficient to determine the relative curative value of the materials heated in acid and alkaline solution respectively, comparing the results obtained with those of the previous work when growth was the criterion. Ten tests were made, six rats being treated with material (*a*) and four with (*b*).

RESULTS OF THE CURATIVE TESTS.

Yeast extract autoclaved in acid solution.

Of six rats treated with doses of this material, three received a daily dose equivalent to 0·5 g. yeast. They all showed marked improvement in a week, and normal appearance of skin and fur was attained in $2\frac{1}{2}$ to 3 weeks (rats 645, 574, 624). Simultaneous with the improvement in the skin condition there was immediate resumption of growth, the average weekly increase in body weight being 10–20 g.

With smaller doses of the same material the result was not so dramatic. Rat 589, which had a severely affected skin after 16 weeks' deprivation of vitamin B_2, received the equivalent of 0·33 g. yeast daily for $4\frac{1}{2}$ weeks. At the end of this time the body weight had increased by 38 g. (from 66 to 104 g.), and the skin and fur, though much improved, were not quite restored to a normal condition. Rats 664 and 564 received the equivalent of 0·16 g. yeast daily. This proved inadequate for complete cures, though the skin condition was improved and growth was resumed.

Yeast extract autoclaved in alkaline solution.

Four rats received this material in doses equivalent to 0·5–1·0 g. yeast daily, for periods of from 3 to 5 weeks; they showed no improvement and in three cases the skin lesions became definitely worse. For example, rat 599, with only a moderate skin affection, received the equivalent of 1·0 g. yeast for 23 days, during which time the symptoms became progressively more severe. Bare, desquamating patches of skin developed round the eyes, and on the neck and shoulders bald areas appeared, which later became raw and inflamed. Diarrhoea also occurred and there was a slight loss in weight (10 g.).

Rat 565 received a dose equivalent to 0·5 g. yeast daily for 21 days during which the skin symptoms became steadily worse, and there was slight loss of weight. Increasing the dose to the equivalent of 1·0 g. yeast was of no use, but, when this was replaced by extract autoclaved in acid solution equivalent to 0·5 g. yeast, a swift improvement took place. In 10 days the skin had assumed a healthy appearance, new fur was growing, the blood-stained discharges from nose and eyes had discontinued and the diarrhoea, which had developed during the preceding period, also disappeared. There was an increase in weight of 27 g. in the 10 days.

Rats 616 and 635 received the material treated in alkaline solution in the equivalent of 0·5 and 1·0 g. yeast respectively. Rat 616 became definitely worse during the 27 days of its treatment and lost 9 g. in weight; rat 635 on the larger dose maintained weight and continued with symptoms unchanged for 5 weeks. The subsequent cure of these two rats took place with material kindly provided by Dr Reader. This material was a solution of marmite autoclaved at 120° for 1 hour at p_H 9·0 and was found by Dr Reader to be effective in curing the characteristic dermatitis due to deficiency of vitamin B_2, but

Heat-stability of vitamin B_2 in yeast extract (XXIV). Autoclaved at 119° for 4 hours. Curative tests. Vitamin B_1 provided as Peters's concentrate.

p_H of extract when heated	Dose, expressed as equivalent of dry yeast g.	Rat no. and sex	Time on "$-B_2$" diet. Weeks	Symptoms: general condition	Body weight g.	Average weekly increase in body weight during treatment g.	Result of treatment
2·5	0·5	574 ♀	16	Eyes badly affected, bloody discharge from nose and eyes. Fur very poor on chest	72	16	Rapid cure. 1st week, eyes and skin became healthy and new fur began. Skin and eyes normal in 2½ weeks
2·5	0·5	624 ♀	12	Skin symptoms severe. Eyes sunken, secreting, "spectacled." Bloody discharge also from nose. Fur very poor, thin and "stringy" over face and neck. Abdomen stained	47	14	Rapid cure. 1st week. Eyes nearly normal, posture also. 2nd week. All symptoms disappeared. 3rd week. Normal fur
2·5	0·5	645 ♀	6	Skin symptoms slight but animal very weak and apparently moribund. Abdomen stained	42	21	Rapid cure. Coat appeared normal in 9 days
2·5	0·33	589 ♀	16	Skin symptoms severe. Eyes "spectacled"; left nearly closed. Fur poor and stringy on face and neck. Bloody discharge from nose and eyes. Abdomen stained	66	9	Slow cure. Eyes improved and stains on fur from discharges disappeared in 3 weeks. New fur growing and coat becoming normal in 4½ weeks
2·5	0·16	604 ♀	12	Eyes "spectacled." Paws blood-stained. Fur poor and "stringy" over face and neck. Abdomen stained. Condition miserable	55	6	Very slow cure. Symptoms arrested in 1st week. Stains on abdomen disappeared in 3rd week, skin and eyes not normal after 4 weeks
2·5	0·16	564 ♀	21	Skin symptoms severe. Eyes "spectacled," nostrils inflamed, bloody discharge. Fur "stringy," bald patches round ears, raw patches round anus and vagina. Abdomen stained. Condition miserable	50	7	Marked improvement in 1 week, ears and forepaws desquamating. Skin round vagina dry and healthy

10·3–9·6	1·0	599 ♂	17	Symptoms slight. Eyes sunken, "spectacled." Bloody discharge from nose. Fur thin on face. Abdomen stained	81	Lost 10 g. in 23 days	No cure. Symptoms became worse in 2–3 weeks. Bare, raw, desquamating patches developed round eyes and on left side of neck and shoulder. Skin round nose and lips became swollen and cracked, area of inflamed skin developed round anus. Animal very miserable. Killed
10·3–9·6	1·0	635 ♂	10	Skin symptoms severe. Eyes sunken, "spectacled." Bloody discharge from nose. Fur "stringy," bald patches round ears. Nose tip inflamed	49	2·5	No cure. Condition remained almost unchanged except for onset of diarrhoea during 5 weeks' treatment. Treated with Dr Reader's preparation of marmite. Marked improvement in 1 week. In 3 weeks coat almost normal, animal in normal health, increase in weight 72 g.
10·3–9·6	0·5 later 1·0	565 ♂	15	Skin symptoms not severe. Eyes sunken and inflamed. Bloody discharge from nose. Diarrhoea	88	Lost 15 g. in 22 days	No cure. Condition became worse. In 3 weeks raw, bald patches were spreading from the axillae down the forearms. Mouth sore and inflamed. Condition of animal miserable. Treated with acid-autoclaved material equivalent to 0·5 g. yeast. Rapid cure. In 10 days nose healing; new fur growing everywhere, increase in weight 27 g.
10·3–9·6	0·5	616 ♂	13	Skin symptoms slight and incipient. General condition fair	56	Lost 9 g. in 27 days	No cure. Condition rapidly grew worse. In 3 weeks inflamed, bare patch of skin developed under chin, nostrils inflamed, fur "stringy." Treated with Dr Reader's preparation of marmite. Marked improvement in 1st week, in 3 weeks nostrils healed, coat almost normal; increase in weight 56 g.

incapable of restoring growth on basal diets lacking in vitamin B_2 and provided with abundant vitamin B_1. In our experiments, not only were the skin lesions rapidly healed but immediate vigorous growth took place. The weight of rat 635, on the dose prescribed by Dr Reader, was increased by 72 g. in 3 weeks and that of rat 616, on about half this dose, by 56 g. in the same time.

This divergence would be explained if our preparation of vitamin B_1 (Peters's concentrate prepared from brewer's yeast) contained Dr Reader's third factor. We are now examining this preparation for evidence of any factor other than the antineuritic vitamin B_1 and are endeavouring to separate it, if present. The results of these researches will be published shortly. At the present time we can only report that we have failed to obtain satisfactory cures of dermatitis unaccompanied by increase in weight. Another possible explanation of the results obtained with Dr Reader's solution is that the material was originally so rich in vitamin B_2 that the residue, after exposure to 120° for 1 hour in alkaline solution, still contained sufficient in the doses given to cure the dermatitis and to stimulate growth in the rats of our experiment.

SUMMARY.

1. An extract made from washed brewer's yeast (XXIV) by boiling in dilute acetic acid, after being heated in acid solution (p_H 2·5) for 4 hours at 119° (solution *a*), retained its power to cure the skin affection in rats due to deprivation of vitamin B_2 swiftly and completely in doses equivalent to 0·5 g. yeast (dry wt.) and more slowly and less completely in doses equivalent to 0·16–0·33 g. yeast.

2. When the same extract was exposed to the same treatment in alkaline solution (p_H 10·3–9·6) (solution *b*), no curative properties could be demonstrated in doses equivalent to 0·5 g. or 1·0 g. yeast and in three out of four cases the skin affection became rapidly worse.

3. Growth of the rats was parallel with the changes in the condition of the skin; when treated with solution (*a*) growth was restored, with solution (*b*) body weight remained stationary or decreased.

In conclusion we wish to thank Dr Reader for providing the material used for the cure of rats 565 and 616, and Messrs Watney, Coombe, Reid & Co. for providing the brewer's yeast from which our extract was made.

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CIV. THE STABILITY OF WATERY SOLUTIONS OF THE OXYTOCIC PRINCIPLE OF THE PITUITARY GLAND.

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(*Received June 20th, 1930.*)

It has long been known that the substance in the posterior lobe of the pituitary gland which produces contractions of the uterus is destroyed by alkalis in the cold [Guggenheim, 1914], is stable in weakly acid watery solution, and is destroyed by boiling in strong acids [Abel and Nagayama, 1920; Dale and Dudley, 1921].

Adams [1917] showed that when weakly acid extracts were heated the oxytocic activity, measured on the guinea-pig's uterus, disappeared from the solution in such a way that its rate of disappearance at any moment was proportional to the activity present at that moment. The solutions were much more stable at p_H 3 than at p_H 5.

This paper deals with a number of determinations which have been carried out, under my direction, by my assistant, L. S. Drewell, with the object of obtaining more complete quantitative knowledge of the stability of this substance. Buffered extracts were prepared from the British standard preparation of the posterior lobe, heated for different times and assayed. The tests were carried out on the virgin guinea-pig's uterus by the method described by Dale and Laidlaw [1912], the details of the apparatus being those described by Burn and Dale [1922]. The results are comparable with, and in many ways similar to, the results obtained by Krogh and Hemmingsen [1928] in their study of the action of heat on insulin. For the sake of uniformity the notation of these authors has been adopted in the present paper.

Methods.

Heating was carried out in ampoules of hard glass, sealed and placed in a water-bath, the temperature of which was maintained constant. This was effected either by means of a toluene regulator, or when, as in most cases, temperatures near to 100° were required, by boiling the water and maintaining its level with an overflow tube—a procedure which produced a constant temperature of about 99°.

Over a wide range of p_H it would be inconvenient to study the rate of destruction at low temperatures, since it would be necessary to keep the

extracts for many months in order to get any considerable destruction. The effect of p_H on the stability has, therefore, been most completely studied at 99°. The application of these results to the stability at lower temperatures is discussed below.

The hydrogen ion concentration of the solutions was buffered where possible with NaOH and NaH_2PO_4 in different proportions [Prideaux, 1911]. Potassium was excluded from the solutions because it would have interfered with the final assay. Between p_H 3 and p_H 5·5, where the buffering of phosphates is slight, Walpole's HCl-acetate mixtures were used. For one measurement at p_H 8·5 Palitsch's boric acid-borate mixture was used [Clark, 1928].

The total concentration of phosphate, acetate or borate was $N/7$, so that these solutions were approximately isotonic with blood. The p_H of the solutions was determined colorimetrically, and in most cases electrometrically, and was found not to differ widely from the p_H calculated from the weights of the salts used in preparing the buffer. Solutions of p_H 1 and p_H 2·1 were obtained by diluting a standard extract in 0·25 % acetic acid with HCl, so that the final concentration of HCl was $N/10$ and $N/100$. Similar solutions in N HCl, N NaOH and $N/10$ NaOH were taken to represent p_H 0, p_H 14, p_H 13. In these very acid and alkaline solutions the initial concentration of the pituitary extract was 1 unit per cc. In all the other experiments it was 2 units per cc.

The measurements of p_H were made at room temperature, whilst most of the experiments were carried out at about 100°. It is improbable that this circumstance introduced any considerable error since the p_H of phosphate buffers [Walbum, 1920], acetate buffers [McIntosh and Smart, 1920; Clark, 1928], and strong acids and bases are said to be practically unaffected by changes of temperature. In all cases the solutions were made faintly acid for testing.

In the case of the observations between p_H 3 and p_H 7·25 extracts were made by boiling the standard powder in the buffer solution for 10 minutes, cooling and filtering. The filtrate was heated to a known temperature for a known time and its activity then determined by comparison with a standard extract in 0·25 % acetic acid. The assumption was made that the extraction was complete, and a small correction was applied for the loss of activity during the initial 10 minutes' boiling. In the case of the observations at p_H 11·1 it was possible in the light of more complete knowledge to adopt a more satisfactory procedure. The extraction was carried out in a solution of acid sodium phosphate. A portion of this extract was kept as a standard solution and another portion was treated with a suitable quantity of NaOH, maintained at a constant temperature for a definite time and then faintly acidified with HCl, cooled, made up to a known volume and tested.

The course of the reaction.

If Adams's [1917] conclusions as to the course of the reaction are correct, the velocity of the change may be measured, like that of the destruction of

insulin, in terms of a constant k which is equal to the rate of fall per hour of $\log_{10} C$ (where C is the concentration of the active principle).

The fact that k remains constant has been confirmed by carrying out two or more experiments at each of seven different values of the p_H in which the time of heating was varied. These results are shown with others in Fig. 1. The values of k showed no regular tendency either to increase or decrease and the variations which occurred were no larger than might have been expected.

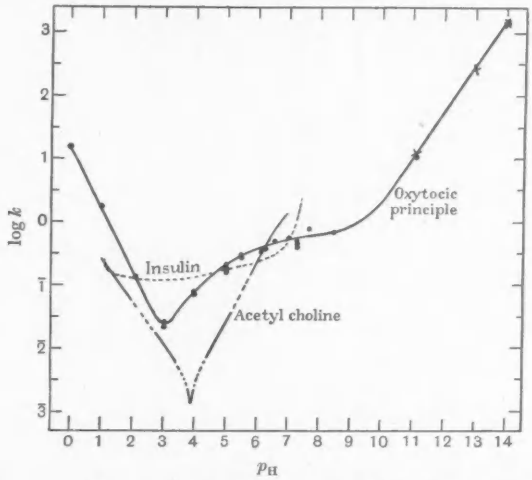

Fig. 1. Abscissae—p_H. Ordinates—log k—where k is rate of destruction at 99°. Dots represent direct observations. Crosses represent results calculated from observations at 25°.

In order to determine k under any particular circumstances it is desirable that the reaction should continue until destruction is nearly complete, since in this case the error of the assay has less effect on the result. The experiments have therefore been planned so that the percentage destruction was always over 50 and usually about 90, but it was, of course, not possible to ensure uniformity in this respect.

The effect of temperature.

The variation of the velocity of the reaction with the absolute temperature T is defined with sufficient accuracy by Arrhenius's equation

$$k_1 = k_0 e^{\frac{\mu}{2}\left(\frac{T_1 - T_0}{T_1 T_0}\right)},$$

where k_1 and k_0 are the rates of destruction at absolute temperatures T_1 and T_0. Measurements have been carried out at p_H 6·55 and at p_H 11·1 and the results are recorded in Table I. In each case three estimates of the constant μ in the above equation have been obtained by combining the result of the experiment at 99° with the results of those at lower temperatures.

Table I.

Temp. °	Time of heating (hrs.)	Activity left %	k (corrected)	μ
		p_H 6·55		
99	1·33	17·6	0·503	—
80	16·0	1·39	0·111	20,900
60	42·0	11·6	0·02	20,500
50	69·2	33·8	0·0056	22,100
		p_H 11·1		
99	0·1	8·0	10·97	—
74·5	0·783	3·0	1·94	18,200
50	10·68	3·3	0·142	21,300
25	47·67	33·3	0·01	21,000

The variations in μ are irregular and are not large considering the methods used. For the purpose of calculation it is assumed that μ has a constant value of 21,300—an estimate of its value which was obtained graphically. These observations have also been taken to justify the assumption that the value of μ is independent of the p_H. The absolute value of μ is low compared with the values obtained for the spontaneous disintegration of various more complex substances [Arrhenius, 1915]. It is lower than the value obtained for the destruction of insulin—28,300 [Krogh and Hemmingsen, 1928], but it is high compared with the values met with in most chemical reactions.

The effect of hydrogen ion concentration.

The variation of k with the p_H is large and in order to get all the readings on the same curve the p_H has been plotted in Fig. 1 not against k but against its logarithm. The readings shown by dots were obtained in the boiling water-bath, the temperature of which was 99°. The readings shown by crosses were obtained at 25° and the values which would have been obtained at 99° were calculated from the data given above and plotted.

In the region of strong acidity the results fall on a straight line the slope of which indicates that the rate of destruction is directly proportional to the hydrogen ion concentration.

In the region of strong basicity the results also fall on a straight line, but in this region the rate of destruction is proportional to $[C_{OH}]^{0·73}$.

The effect of variation of the p_H on the stability is at a minimum between p_H 7 and 8.

For the sake of comparison similar curves for insulin and for acetylcholine are shown. The former is taken from the paper by Krogh and Hemmingsen [1928]. The latter was obtained by combining some observations published by Hofmann [1930] with some unpublished observations which Dr K. Mathes has kindly allowed me to use.

One experiment was carried out to test the possibility that the rate of destruction was considerably affected by the amount of salt in the solution. It was found that when the concentration of phosphate at p_H 7·2 was divided by ten, the other factors remaining constant, the value of log k fell by 0·071.

It was concluded that if any salt effect is present it is small compared with the other factors and it has been neglected.

All the results are summarised in Fig. 2.

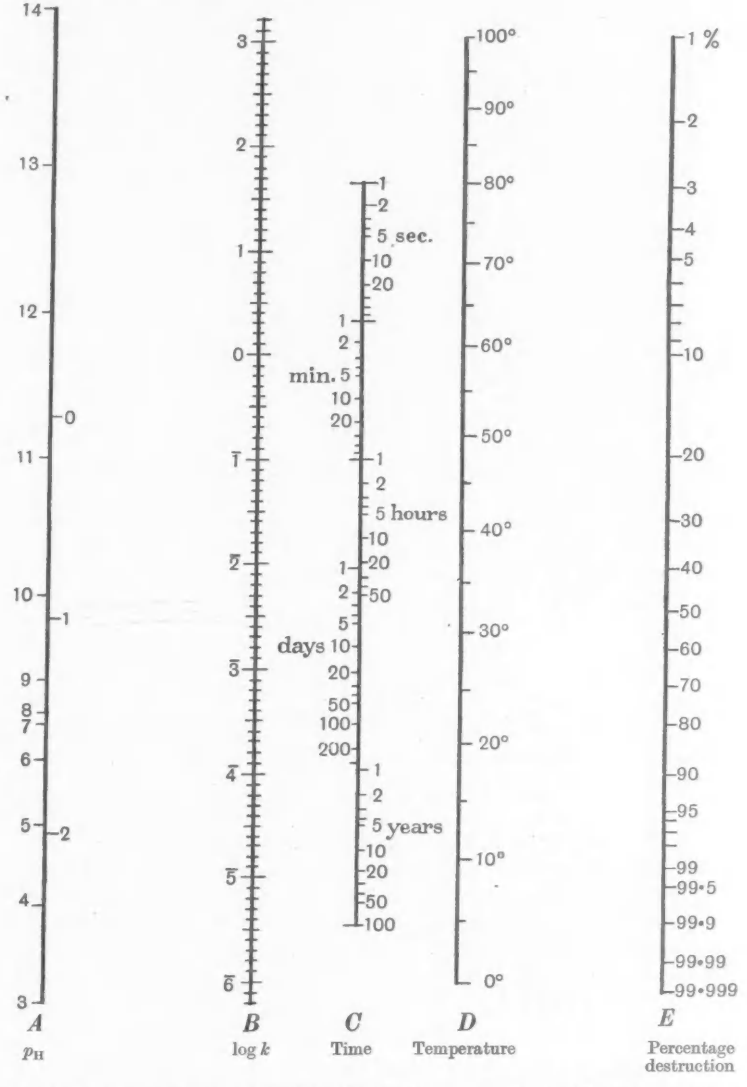

Fig. 2. Log k is determined on scale B by aligning the p_H on scale A with the temperature on scale D. The percentage destruction is obtained on scale E by aligning log k on scale B with the time on scale C.

The use of this nomogram is most readily explained by taking an example. The p_H of the standard extract in 0·25 % acetic acid is about 4 [Kamm *et al.*, 1928]. If a thread be stretched from p_H 4 on scale *A* to 100° on scale *D* it will be found that when this solution is boiled $\log k = \bar{2}{\cdot}9$. By stretching the thread through $\bar{2}{\cdot}9$ on scale *B* and 10 minutes on scale *C* it is found that when this solution is boiled for 10 minutes about 3 % of the oxytocic principle is destroyed. Similarly, if this solution is kept for a year at 0° the loss of activity is about 5 % and would probably not be detected. This last conclusion has been confirmed.

It should be emphasised that these results were obtained with extracts of a powder which contained all the substances present in the posterior lobe of the pituitary which are soluble in water, but not in acetone. It is possible that preparations that are either more or less pure may give different results. It has, however, been found that the stability of the preparation known as "pitocin" or "oxytocin" [Kamm *et al.*, 1928], at p_H 7 and 99°, is identical, within the error of the method, with that of the oxytocic substance in the comparatively crude extracts used in the present experiments.

Summary.

The rate of destruction of the substance in watery extracts of the standard preparation of the posterior lobe of the pituitary gland, which causes contraction of the guinea-pig's uterus, has been measured at different hydrogen ion concentrations and at different temperatures. The results are summarised in a nomogram (Fig. 2) which gives the amount of destruction to be expected when a solution of any given p_H is heated for any given time at any given temperature.

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CV. SYNTHESIS OF THE *i*-HYDROXY-ASPARAGINES.

By ALBERT CHARLES CHIBNALL.

A NOTE ON THEIR DISSOCIATION CONSTANTS.

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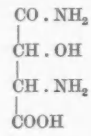
THE important part played by acid amides in the protein metabolism of the plant is too well known to require emphasis: these substances have long attracted the attention of plant chemists on account of the relative ease with which asparagine and glutamine—the only amides which have yet been found in natural products—can be isolated from plant extracts. It is perhaps less well known that the amount of these two amides which can be obtained in crystalline form from any given plant extract is always less than that which the "amide-nitrogen," as determined by Sachsse's method [1873], suggests should be present. Two explanations of this readily suggest themselves. In the first place the presence of other soluble plant products prevents the crystallisation of the total amount of asparagine or its copper salt. Where the amount of these other products is small, as in intensely etiolated lupin seedlings, Schulze [1880] obtained a yield of 90 % of crystalline asparagine; but in less intensely etiolated plants, or in the normal green plant wherein the other products predominate, the yield is always low and seldom exceeds 30 %. In the second place, it may well be that amides other than asparagine or glutamine are present in the plant, and that these have so far escaped detection because they possess different solubility properties from the other two amides. Schulze admitted this alternative, which appears the more probable from Vickery's [1924] recent work on the water-soluble nitrogenous materials of green alfalfa plants. Vickery's results suggest that the possible unknown amides were similar to asparagine and glutamine, *i.e.* they were monoamides of monoaminodicarboxylic acids. As on theoretical grounds it would seem possible that one of these might be a hydroxyasparagine, the synthesis of the *i*-hydroxyasparagines was undertaken so that future search for one of the possible new amides in plant products could be guided by a knowledge of the chemical and physical properties of these substances.

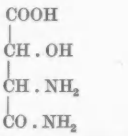
The synthesis presented in some ways the same difficulties which confronted

Piutti [1888] when he worked out the constitution and synthesis of the α- and β-asparagines. These arose chiefly from the fact that the action of hot alcoholic ammonia on the diethyl ester of aspartic acid did not give the expected diamide, but a ring product, "aspartimide," which was afterwards shown by Fischer to be the diamide of 2 : 5-diketopiperazine-3 : 6-diacetic acid. In the present instance it was found that the action of hot alcoholic ammonia on the diethyl ester of the *anti*-acid led to considerable decomposition, and no definite substance could be isolated from the resulting dark brown oil. The synthesis of the diamide was, however, readily accomplished by the method of Fischer and Königs [1904], who showed that the diethyl ester of *l*-aspartic acid and fluid ammonia at room temperature reacted to produce the diamide of *l*-aspartic acid (yield about 50 %) and "aspartimide." This diamide on standing with cold aqueous baryta gave a good yield of *l*-β-asparagine.

i-para- and *i-anti*-Hydroxyaspartic acids were synthesised from maleic acid without difficulty by Dakin's [1921] method and reacted with fluid ammonia to give the diamide in good yield, together with other products which probably correspond to "aspartimides." When the diamide was allowed to stand with cold aqueous baryta partial hydrolysis occurred, with the formation of both the α- and β-hydroxyasparagines.



β-Hydroxyasparagine
(1)



α-Hydroxyasparagine
(2)

The α- and β-hydroxyasparagines are readily differentiated by means of their copper salts. The β-amide (1) gives a normal copper salt containing one equivalent of the metal, whereas the α-amide (2), having the hydroxyl group contiguous to a carboxyl group, takes up two equivalents, indicating that the hydrogen of the hydroxyl has been replaced by metal. Similar abnormal salts are given by hydroxyaspartic acid itself, by *iso*serine, and, as is well known, by malic and tartaric acids. Confirmatory evidence of the position of the amide group in these two amides is given by the biuret reaction. Schiff [1900] showed that the colour developed depends on the number of carbon atoms separating the NH_2-groups. The amide which the copper salt suggests is α-hydroxyasparagine gives a red-violet (oxamide type) colour, showing that the NH_2-groups are attached to adjacent carbon atoms, while the β-hydroxyasparagine gives a blue-violet (malonamide type) colour, showing that the NH_2-groups are connected through a chain of three carbon atoms. Similar colour reactions are given by α- and β-asparagine respectively.

The α-hydroxyasparagines crystallise with 1 H_2O in well-defined stars and long tapering needles, somewhat resembling glutamine. They are readily distinguishable from *l*-β-asparagine. The crystals of the β-hydroxyasparagines

however are anhydrous, and under the microscope exhibit rhombic hemihedral forms which are indistinguishable from those of *l*-β-asparagine. Unlike the latter they exhibit no tendency to form large crystals on slow evaporation of an aqueous extract, a difference which is still apparent when a mixture of the two is allowed slowly to crystallise.

With regard to the general properties of the hydroxyasparagines, the α- and β-forms, as mentioned above, are readily distinguishable by their differences in water of crystallisation, crystalline form, and copper content of the copper salts. They also exhibit different dissociation constants. No differences, however, have been noted—except perhaps slight ones in solubility—between the corresponding *anti*- and *para*-forms.

Both the α- and β-hydroxyasparagines reduce permanganate fairly readily in neutral solution, an observation which can be used to distinguish them readily from *l*-β-asparagine. They give no reaction with Fenton's reagent for tartaric acid, and their colour reactions with various phenols and sulphuric acid do not differ in any marked way from those given by *l*-β-asparagine. Like the latter, also, they have no sharp melting or decomposition point: when heated they slowly darken above 175° and decompose between 230° and 250°.

As to the possible presence of a hydroxyasparagine in plant products the following observations may be of use.

(1) With mercuric nitrate they give heavy white precipitates similar to that given by *l*-β-asparagine.

(2) The copper salts are practically insoluble in cold water, but are fairly readily soluble in boiling water in contradistinction to the copper salt of *l*-β-asparagine, which is soluble only with difficulty in boiling water. The copper salts of the hydroxyasparagines are soluble in excess of copper acetate. Boiling with excess of freshly precipitated cupric hydroxide or with copper carbonate is therefore recommended in any attempt to isolate these salts from plant extracts. Differences in the colours of these various salts which are noted below may prove, in practice, to be somewhat deceptive, as the exact shade of blue varies somewhat with the degree of fineness of the crystals.

(3) If one equivalent of aqueous baryta be added to a solution of any hydroxyasparagine and carbon dioxide passed, no precipitate of barium carbonate occurs, even on boiling, indicating the presence of either an abnormal barium salt or carbamate. Sufficient material has not been available further to investigate this interesting observation.

(4) The α-hydroxyasparagines crystallise so readily and have such a small solubility in cold water that it is improbable that they would have escaped detection had they been present to any extent in plant extracts. The β-hydroxyasparagines, however, are much more soluble in water, and have been found in certain cases to crystallise only with great difficulty in the presence of much foreign material. They might well be present in the syrupy mother liquors which are known to contain so much of the unaccounted "amide-nitrogen" of plant extracts.

(5) Plant chemists occasionally identify asparagine by its crystalline form and nitrogen content, and also glutamine by the copper content of its copper salt. It may not be out of place to call attention to the fact that for all practical purposes β-hydroxyasparagine ($C_4H_8O_4N_2$) and β-asparagine ($C_4H_8O_3N_2$, H_2O) have the same crystalline form and nitrogen content, and that the copper content of the copper salt of β-hydroxyasparagine is the same as that of the copper salt of glutamine.

Experimental.

i-*Hydroxyaspartic acids*. Dakin's method [1921] was followed in all essential details. Maleic acid was used as the starting material for the synthesis of bromomalic and chloromalic acids, which were then heated with strong ammonia at 100° for 10 hours to give a mixture of the *i*-hydroxyaspartic acids. These were separated by fractional crystallisation.

i-para-*Hydroxyaspartic acid diethyl ester*. 6 g. of finely powdered acid were suspended in 50 cc. of absolute alcohol and esterified in the usual way with hydrogen chloride. The ester hydrochlorides were decomposed with K_2CO_3, the ethereal solution of the esters dried with anhydrous sodium sulphate and the ether removed in high vacuum at 30°. The ester was a pale yellow viscous oil. Yield 5·5 g. or 70 % of theory. No attempt was made to purify the product by distillation *in vacuo*, as Dakin states that this leads to considerable decomposition. (Found: C, 46·1; H, 7·2; N, 6·8. $C_8H_{15}O_5N$ requires C, 46·8; H, 7·3; N, 6·8 %.)

i-para-*Hydroxyaspartic acid diamide*. 6·3 g. of the diethyl ester were placed at the bottom of a thick-walled Carius tube, which was then cooled by solid carbon dioxide and acetone. Three to four volumes of fluid ammonia were then run in and the tube sealed. The tube was removed from the bath, allowed to attain room temperature and gently shaken to mix the ester and fluid ammonia. At the end of the first day small white crystals appeared in the liquid, and at the end of the fourth day, when the tube was opened, the walls of the tube in contact with the fluid were covered with a crystalline mat. The fluid ammonia was allowed to evaporate slowly. The resulting yellow crystalline mass was scraped out of the tube, powdered, extracted twice with 40 cc. of warm ethyl alcohol to remove unchanged ester and dried *in vacuo* over solid sodium hydroxide. Yield 4·2 g. Found: N, 27·1 %; $C_4H_9O_3N_3$ requires N, 28·6 %, so that products other than the diamide had been formed. These could not be separated by extraction with warm ethyl alcohol as in the case of the corresponding *anti*-preparations, for the diamide is itself somewhat soluble in this reagent. The mixture was very soluble in cold water, but on standing for some days a small amount of crystalline diamide separated. Recrystallised from a little hot water it was obtained in well-formed cubes and rectangular plates. (Found: C, 32·5; H, 6·1; N, 28·7. $C_4H_9O_3N_3$ requires C, 32·6; H, 6·1; N, 28·6 %.) Heated in an open capillary tube it darkens slowly above 170°, shrinks at 173° and melts with decomposition and evolution

of gas at 174° (corr.). The diamide reacts alkaline to litmus, and, as one would expect, gives an intense red-violet biuret reaction. It is freely soluble in warm water, and when pure is much less soluble in cold water. It is somewhat soluble in warm methyl and ethyl alcohols, only slightly soluble in the cold solvents or in acetone, and is insoluble in ether.

It gives a heavy white precipitate with mercuric nitrate, soluble in excess of nitric acid and a precipitate with mercuric chloride which dissolves on warming and reappears on cooling. It gives a small precipitate with phosphotungstic acid on standing, but none with chloroplatinic acid or with concentrated sulphuric acid. The aqueous solution on boiling slowly gives off ammonia. When 0·35 g. dissolved in 350 cc. of water was boiled gently in a flask about one-quarter of the total nitrogen was removed in about 16 hours. The solution slowly darkened and on allowing the residual liquid to evaporate slowly to a syrup in a desiccator a dark brown gum, similar to that given by the action of hot alcoholic ammonia on the ester, was obtained. The action of a mild alkali, such as baryta, results in the splitting off of one amide group, with the liberation of one-third of the total nitrogen as ammonia and the formation of a mixture of the two monoamides.

i-*α*-para-*Hydroxyasparagine.* 3·5 g. of the crude *para*-diamide were dissolved in a little water and placed in a Van Slyke apparatus for determining amide-N *in vacuo*. Water containing 6 g. of crystalline baryta was then added, and the total volume made up to 70 cc. At the end of 16 hours the ammonia set free was distilled off *in vacuo*, the residual liquor filtered to remove a small white deposit, which probably contained barium *para*-hydroxyaspartate, and barium removed exactly with sulphuric acid. The resulting solution was concentrated to 40 cc. *in vacuo* and allowed to stand overnight. 0·85 g. of crystalline material, consisting chiefly of tapering needles and complicated stars, was removed. On standing for a further 36 hours another 0·29 g. of similar material was obtained. The combined crystalline fractions were dissolved in 45 cc. of hot water, filtered from a small amount of insoluble residue and allowed to crystallise slowly. The resulting product, when viewed under the microscope, was seen to consist almost entirely of tapering prisms and stars, with only occasional plates of the *β*-amide. After two further crystallisations from water a product (0·8 g.) free from the *β*-amide was obtained. It crystallises from water in fine tapering prisms and stars of complicated form, and contains 1 H_2O which is only removed in high vacuum at 100°. (Found: C, 28·7; H, 5·9; N, 17·1; H_2O, 10·8. $C_4H_8O_4N_2$, H_2O requires C, 28·9; H, 6·0; N, 16·9; H_2O, 10·8 %.) It is freely soluble in boiling water, and crystallises readily from solution on cooling. At 18° it is soluble in about 125 parts of water. If a solution of the amide is first neutralised by the addition of one equivalent of sodium hydroxide and excess of a strong solution of copper acetate added, the solution turns a deep blue and becomes strongly acid to litmus, owing to the liberation of acetic acid and the formation of an abnormal salt containing two equivalents of copper. It crystallises readily in microscopic

aggregates of stars and needles containing 1 H_2O, which is not readily removed at 105°. (Found: H_2O, 8·0. $Cu(C_4H_6O_4N_2)$, H_2O requires H_2O, 7·9 %.) For analysis the material was dried *in vacuo* over P_2O_5 at 100°. (Found: C, 23·0; H, 3·1; N, 13·1; Cu, 29·6. $Cu(C_4H_6O_4N_2)$ requires C, 22·9; H, 2·8; N, 13·4; Cu, 30·4 %.)

The copper salt, which can be prepared equally well by boiling with freshly precipitated and well-washed cupric hydroxide, or by the addition of copper acetate without the previous addition of sodium hydroxide, is almost insoluble in cold water, but is moderately soluble in boiling water, from which it can be readily recrystallised. The blue colour of the salt is a little paler than that of crystalline copper sulphate and is easily distinguished from that of the corresponding *β*-hydroxyasparagine and also of *l*-*β*-asparagine.

i-*β*-para-*Hydroxyasparagine.* The mother liquor from the isolation of the corresponding *α*-monoamide mentioned above was allowed to evaporate slowly in a desiccator over sulphuric acid. When the volume had been reduced to less than 5 cc. crystals appeared, and after several days the syrupy residue had set nearly solid. It was filtered at the pump and washed with a little cold water. The white crystalline mass (0·9 g.) was dissolved in 20 cc. of warm water and allowed to evaporate slowly in a desiccator. The first two crops of crystals consisted chiefly of hexagonal plates, and on repeated recrystallisation from water there was finally obtained a fraction (0·4 g.) which appeared under the microscope to be uniform, consisting of simple and complicated rhombic plates and prisms, which are indistinguishable from the corresponding forms of *l*-*β*-asparagine. The *β*-monoamide contains no water of crystallisation. (Found: C, 32·2; H, 5·5; N, 18·6; amide-N [Sachsse, 1873], 9·3. $C_4H_8O_4N_2$ requires C, 32·4; H, 5·4; N, 18·9; amide-N, 9·4 %.) The *β*-monoamide dissolves in about 55 parts of cold water and is freely soluble in boiling water. In the presence of each other the *α*- and *β*-monoamides crystallise out readily in their respective forms, but in the presence of other substances their solubility, especially that of the *β*-monoamide, is greatly increased, and crystallisation is slow. The *β*-monoamide and *l*-*β*-asparagine crystallise readily in the presence of each other in crystals that appear uniform and may be mixed. The *α*-monoamide also crystallises readily in the presence of *l*-*β*-asparagine, but the two crystalline forms are distinct, and a tendency to form mixed crystals was not observed.

The copper salt is prepared in a similar way to that of the *α*-amide. It separates in small amorphous spheres, which, under high magnification, are seen to consist of star-shaped aggregates of stumpy needles. It contains one equivalent of copper, and 1 H_2O which is not completely removed in an oven at 105°. (Found: H_2O, 4·8. $Cu(C_4H_7O_4N_2)_2$, H_2O requires H_2O, 4·8 %.) For analysis the material was dried *in vacuo* at 100° over P_2O_5. (Found: C, 26·8; H, 4·1; Cu, 17·8; N, 15·8. $Cu(C_4H_7O_4N_2)_2$ requires C, 26·8; H, 3·9; Cu, 17·8; N, 15·7 %.)

The synthesis of the amides of the *i*-*anti*-hydroxyaspartic acid was similar

to that described for the *para*-acid above, consequently only points of difference in the two series and necessary analyses are recorded.

i-anti-*Hydroxyaspartic acid diethyl ester*. This acid resembles glutamic acid in that a relatively large amount of alcohol is necessary to effect its initial solution. 6 g. of the finely pulverised acid were suspended in 60 cc. of absolute alcohol, and a rapid stream of dry hydrogen chloride was passed in until the alcohol boiled. The boiling was continued for 1 hour on the water-bath, but much acid still remained undissolved. The mixture was therefore cooled, and the dry gas again passed until the alcohol boiled. The boiling was continued for another hour, when complete solution of the acid was obtained. The ester hydrochloride, though fairly soluble in alcohol, is very insoluble in cold alcohol containing hydrogen chloride. Yield of ester, a pale yellow oil, 4·7 g. or 60 % of theory. It was not distilled. (Found: C, 46·4; H, 7·5; N, 6·6. $C_8H_{15}O_5N$ requires C, 46·8; H, 7·3; N, 6·8 %.)

i-anti-*Hydroxyaspartic acid diamide*. 4·5 g. of the ester were treated with fluid ammonia. The yield of crude diamide was 3·2 g. This was purified by extracting twice with 150 cc. of boiling absolute alcohol. The material (2·5 g.) contained 28·2 % N, and was therefore practically pure *anti*-acid diamide. A small amount of this was dissolved in hot water. On standing overnight it crystallised in small cubes and irregular oblong prisms. (Found: C, 32·7; H, 6·2; N, 28·6. $C_4H_9O_3N_3$ requires C, 32·6; H, 6·1; N, 28·6 %.) Heated in an open tube it commences to darken above 170°, shrinks at 176°, and melts with decomposition at 176·5° (corr.). It gave similar reactions to the *para*-diamide.

i-α-anti-*Hydroxyasparagine*. 2·1 g. of the diamide were dissolved in 70 cc. of water containing 4 g. of crystalline baryta and treated as in the case of the *para*-diamide. Barium was removed, the filtrate concentrated to 20 cc. and on standing overnight 0·8 g. of crystalline material separated. Recrystallised once from warm water it was obtained in blunted needles and prisms, free from the β-monoamide. It contains 1 H_2O which is not removed at 105°. (Found: H_2O, 10·8; amino-N, 8·4. $C_4H_8O_4N_2$, H_2O requires H_2O, 10·8; amino-N, 8·4 %.) It dissolves in about 95 parts of cold water and is freely soluble in boiling water. The copper salt is prepared in a similar way to that of the *para*-amide, and has a similar appearance and solubilities. (Found: C, 20·8; H, 3·6; Cu, 28·3; H_2O, 8·0. $Cu(C_4H_6O_4N_2)$, H_2O requires C, 21·0; H, 3·5; Cu, 28·0; H_2O, 7·9 %.)

i-β-anti-*Hydroxyasparagine*. The mother liquor from the isolation of the α-monoamide described above was allowed to evaporate slowly in a desiccator. Crystallisation was slow, but after some days 0·47 g. had separated and was removed. Redissolved in 10 cc. of warm water it crystallised slowly in needles and stars of the α-monoamide and a few plates of the β-monoamide. After a tedious fractionation there was finally obtained 0·1 g. of material which consisted entirely of the rhombic plates characteristic of the β-monoamide. It contained no water of crystallisation. (Found: C, 32·8; H, 5·6; N, 18·0. $C_4H_8O_4N_2$ requires C, 32·4; H, 5·4; N, 18·9 %.) It is readily soluble in hot water,

and has about the same solubility in cold water as the corresponding α-monoamide. The copper salt separates in nodular aggregates like those of the *para*-β-monoamide, containing one equivalent of copper and 1 H_2O. (Found: H_2O, 5·2. $Cu(C_4H_7O_4N_2)_2$, H_2O requires H_2O, 4·9 %.) For further analysis the material was dried in high vacuum over P_2O_5. (Found: C, 27·0; H, 4·2; Cu, 18·0. $Cu(C_4H_7O_4N_2)_2$ requires C, 26·8; H, 3·9; Cu, 17·8 %.)

Dissociation constants.

Complete hydrogen electrode titrations of aspartic acid, hydroxyaspartic acid, *i*-β-asparagine, *i*-β-*para*-hydroxyasparagine and *i*-α-*anti*-hydroxyasparagine have been carried out. From the data the "titration constants" of each substance have been calculated. These values must be close approximations to the dissociation constants uncorrected for activity ($p_{K'}$) and will be assumed to be identical with them. The mean values are assembled in Table I. Following Brønsted [1923], all constants have been formulated as hydrion dissociation constants. Consequently, the subscript numeral indicates only the order in which the several constants of a substance appear on the p_H scale. If we adopt the zwitterion hypothesis of Bjerrum [1923], assignment of the various constants may be made as follows:

$p_{K_1'}$, the first carboxyl of the dibasic acids and the carboxyl of the amides;

$p_{K_2'}$, the second carboxyl of the dibasic acids and the amino-group of the amides;

$p_{K_3'}$, the amino-group of the dibasic acids.

A number of determinations of the dissociation constants of aspartic acid have appeared in the literature. Some of these, recalculated to conform to the above treatment, are included in the table. Agreement is fair. We have found only one previous determination for β-asparagine. The method employed was that of measurement of the rates of ester hydrolysis. The values so obtained agree well with our results by the more precise electrometric method. The constants of the remaining substances have been determined for the first time. The assembled results form an interesting contribution to the problem of the effect of substitution upon ionisation. They do not, however, cover a sufficient number of cases to make profitable a detailed analysis from this point of view. Moreover, simple effects of substitution are undoubtedly complicated, in substances such as these, by powerful electrostatic forces between ionising groups. It is clear, however, that both the introduction of hydroxyl and the substitution of the hydroxyl group in carboxyl by amide increases the hydrion dissociation of both carboxyl and amino-groups. The magnitude of these effects is reduced to about one-third as the substituent is removed from the α- to the β-position. It is interesting to compare the influence of α-amide formation with that of peptide formation. Comparison with the constants of a typical amino-acid and its dipeptide indicates that these effects upon the amino-group are similar in sign and in magnitude.

Table I.

	COOH	COOH	NH_2	Mol. conc.	Temp. ° C.	Author
	pK_1'	pK_2'	pK_3'			
Aspartic acid	1·90	—	9·47	—	25	Levene and Simms [1925]
	2·08	3·94	9·85	—	—	Hopfield *et al.* [1920]
	2·08	3·66	9·85	—	—	Harris [1924]
	2·05	3·76	9·85	0·02	25	Chibnall and Cannan
Hydroxyaspartic acid	1·95	3·47	9·03	0·02	25	Chibnall and Cannan
	pK_1'		pK_2'			
β-Asparagine	2·18	—	8·87	—	—	Lunden [1906]
	2·02	—	8·80	0·02	25	Chibnall and Cannan
β-Hydroxyasparagine	2·12	—	8·26	0·01	25	Chibnall and Cannan
α-Hydroxyasparagine	2·31	—	7·17	0·01	25	Chibnall and Cannan
Glycine	2·42	—	9·74	—	25	Quoted by Kirk and Schmidt [1929]
Glycylglycine	3·15	—	8·10	—	25	

Experimental.

The technique of titration followed that of Cannan and Knight [1927]. The temperature was accurately maintained at 25°. The reference electrode was a saturated calomel half-cell in electrical contact with the hydrogen electrode vessel through a saturated potassium chloride bridge. The standard of reference was 0·1 *M* hydrochloric acid which was assumed to have a hydrogen electrode potential of 0·0643 v. at 25°. The potentials of the liquid junctions, which were established in a reproducible fashion, were ignored. A pair of palladinised gold-plated platinum wires served as hydrogen electrodes. In no observation did they differ from one another by more than 0·2 mv. The calculation of "titration constants" followed the method of Simms [1926].

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CVI. THE EFFECT OF CRYPTORCHIDISM AND OF CASTRATION ON THE CHEMICAL COMPOSITION OF RATS.

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This investigation not only aimed at ascertaining whether cryptorchidism and castration produce any change in the chemical composition of the tissues of the rat, but was also a trial of a simple method of obtaining approximate, but accurate, information about the general chemical composition of the bodies of such small animals as mice, rats or guinea-pigs. It was mainly desired, in this method, to avoid a long and difficult treatment of the whole body of the animal for analysis. In the present paper only the amount of water, solids, nitrogen and fat in the tissues of the rats was investigated.

Historical.

Reference has been made in previous papers [Korenchevsky, 1914, 1925, 1928, 1930; Korenchevsky and Carr, 1925; Korenchevsky and Schultess-Young, 1928] to the whole literature on castration and cryptorchidism. It is perplexingly contradictory. The fact which is most definitely established is that in most castrated animals and human beings an increase in the body-fat is observed when the testes are removed or are atrophied. Korenchevsky's [1930] experiments on rats confirmed this quite definitely, as well as showing that most cryptorchid animals also become obese, although to a lesser degree than the castrates.

As far as we know there is only one investigation on this subject, by Lüthje [1902, 1903], who studied the chemical composition of whole castrated animals, namely, of two castrated dogs as compared with that of their normal litter mates. Lüthje's technique was perfect, but unfortunately the number of animals used in the experiments, owing to the difficulty of technique, was insufficient for any conclusions. Nevertheless, we refer to these experiments at length, since, considered in connection with our experiments, Lüthje's results will assist in the drawing of our conclusions. The following interpretation of Lüthje's results is ours and was not suggested by him. Lüthje's figures have been recalculated by us as percentages of the final body-weight of the animals and as fat-free body-weight; and are shown in Table I.

Table I. *Percentage content of solids, fat and nitrogen in two castrated dogs as compared with that of normal litter mates.*

	Final weight of dog in g.	Solids %	Fat %	Nitrogen %	Nitrogen per 100 g. fat-free body-weight
Normal ♂	17,650	46·5	9·2	3·01	3·31
Castrated ♂	16,600	47·5	8·6	3·14	3·44
Normal ♀	11,400	45·3	9·15	2·93	3·22
Castrated ♀	12,620	48·1	12·8	2·81	3·23

As was stated in our experiments on dogs [Korenchevsky, 1925], only some of these animals became obese after castration. Similarly in Lüthje's experiments one dog (male) became a "thin" castrate after castration, whilst the bitch was a typical "fat" castrate. Consequently, the results of chemical investigation were different, the general nitrogen content being slightly greater (by 4·3 %) in the "thin" castrate, whilst in the castrated bitch it was slightly less (by 4·1 %), than in the respective control animals. This decrease of nitrogen content in the obese bitch was not evident when the nitrogen content was recalculated per 100 g. of fat-free body-weight. This decrease of nitrogen in the "fat" castrate is therefore only apparent and due to the increased proportion of fat in the body and not to an impoverishment in protein substances of the castrated organism. There was, moreover, a slight increase in the nitrogen of the "thin" castrated male dog. It was shown in a previous paper [Korenchevsky, 1925] that, after castration, the deposition of nitrogen in the body of most castrated animals increases. This increase, indicated by metabolism experiments, agrees well with an increased nitrogen content of the body of the "thin" castrate found in Lüthje's experiment (Table I). An increase in the solids in the bodies of both castrated dogs (see Table I) can be explained by the deposition either of fat, or of nitrogen, or of both.

Present experiments.

The experiments were performed on 46 rats, belonging to 9 litters (see Table II). Each litter was divided into a group of control rats and groups of cryptorchid and castrated rats. The cryptorchids of some litters were divided into one-sided cryptorchids, in which only one testicle was made cryptorchid, and into both-sided cryptorchids in which both testicles were made cryptorchid. Altogether 18 control rats, 5 one-sided cryptorchids, 16 both-sided cryptorchids and 7 castrates were used. All these rats have been described elsewhere [Korenchevsky, 1930] and all the details of the general technique employed are there given, as well as a description of the influence of cryptorchidism and castration on growth, fat deposition, sexual and endocrine organs. In the present investigation, it was decided to use the hind legs for analysis, since they are suitable as a general indication of the chemical composition of the principal tissues of the body, namely, skin (with fur), muscles and skeleton. Two controlling chemical analyses are obtainable, so that the results obtained on one

Table II. *The percentage content of nitrogen in each dry hind leg of each rat.*

	Normals		Cryptorchids: one-sided		Cryptorchids: both-sided		Castrates	
No. of litter	No. of rat	Nitrogen content	No. of rat	Nitrogen content	No. of rat	Nitrogen content	No. of rat	Nitrogen content
5	829	10·21	835	9·88	831	9·58	834	8·92
		10·25		10·48		9·63		8·88
	830	9·90			832	10·24		
		9·89				10·14		
Average		**10·06**		**10·18**		**9·90**		**8·90**
6	836	9·80	838	9·32	839	9·28		
		9·75		8·84		8·96		
	837	9·61			840	—		
		9·58						
	841	9·83						
		9·69						
Average		**9·71**		**9·08**		**9·12**		
7	842	—	843	10·01	844	9·50		
				10·26		9·53		
	845	10·09			846	10·17		
		10·11				10·04		
Average		**10·10**		**10·14**		**9·81**		
8	863	9·60	865	9·45	866	9·07	868	9·20
		9·65		9·73		8·91		9·30
	864	10·01						
		9·86					867	8·77
								8·39
Average		**9·78**		**9·59**		**8·99**		**8·92**
9	869	9·04	872	9·99	871	9·55	873	8·94
		9·22		9·65		9·47		8·82
	870	9·57					874	9·42
		9·74						9·62
Average		**9·39**		**9·82**		**9·51**		**9·20**
22	847	9·78			849	9·35	851	9·12
		9·75				9·29		8·94
	848	9·49					852	9·59
		9·43						9·63
Average		**9·61**				**9·32**		**9·32**
23	856	9·85			854	10·10		
		9·87				9·87		
					855	9·84		
						9·91		
					857	9·73		
						9·70		
Average		**9·86**				**9·86**		
24	858	—			861	9·73		
						9·75		
	859	11·23			862	9·62		
		10·54				9·87		
Average		**10·89**				**9·74**		
25	875	9·68			880	9·28		
		9·53						
	877	10·04			881	10·13		
		10·11				10·18		
Average		**9·84**				**9·86**		
Average of averages of litters		**9·92**		**9·76**		**9·57**		**9.09**

leg can be used to check the results obtained with the other. It was decided to investigate the amount of water, solids and nitrogen in the legs, but it is obvious that any other necessary chemical analyses could also be made. For the approximate estimation of the deposition of fat in the body, a still simpler method, which was used by us in the previous paper [Korenchevsky, 1930], was chosen. In this method, the weight of the retroperineal and testicular fat, which can be quickly and easily separated from the body by means of a scalpel, was taken for the comparative estimation of the degree of deposition of fat in the animal. The comparative changes in fat deposition in different animals can be checked in each animal by the separate weighing of the testicular and retroperineal fats. Elsewhere [Korenchevsky, 1930], only the total amount of intra-abdominal fat (*i.e.* the retroperineal plus testicular) is given. Therefore in the present paper we give, in Table III, the weights of retroperineal fat and of

Table III. *Average weight of retroperineal and testicular fat in groups of normal, cryptorchid and castrated rats.*

	Normal rats		One-sided cryptorchids		Both-sided cryptorchids		Castrates.
No. of litter	Retro-perineal fat	Testi-cular fat	Retro-perineal fat	Testi-cular fat	Retro-perineal fat	Testi-cular fat	Retro-perineal fat
5	6·27	5·70	8·60	8·24	4·13	6·68	12·91
6	6·41	6·97	8·59	9·77	10·02	7·98	—
7	6·96	6·54	6·81	8·49	5·22	9·21	—
8	6·47	8·40	8·28	9·97	8·07	7·95	11·41
9	8·68	7·18	6·02	6·78	8·89	9·91	9·36
22	6·97	5·98	—	—	8·15	9·96	9·67
23	5·27	5·79	—	—	7·35	4·98	—
24	4·32	5·35	—	—	7·74	8·48	—
25	5·77	4·94	—	—	6·66	8·95	—
Average	6·35	6·32	7·66	8·65	7·36	8·23	10·84

testicular fat separately, so that both data can be compared. The weight of fat is calculated in the table as g. per 200 g. body-weight of the rat. The separation of the legs from the body was performed in the following way: the hind leg was bent at the fibula-tibia-femoral junction by the left hand of the investigator and the blades of the scissors placed round and just a little above this junction, in such a manner that the lower blade of the scissors was placed in the angle made by bending the leg at the junction; then the leg was cut off, without producing tension of the skin and muscles and the cut made neat with the scissors. It was possible, without any special training, to sever the legs so accurately, that the dry weight of the right leg of the animal differed from that of the left leg of the same animal, on the average, by only $\pm$ 2·6 % (minimum $\pm$ 0·2, maximum $\pm$ 9·1 %). The legs were placed in weighing bottles and weighed, first fresh and then after desiccation at about 103°. The nitrogen was estimated by the Kjeldahl method in each leg separately. In order to save space only one table, No. II, is given in detail. In all the other tables the changes obtained in groups of litters, and not those in individual rats, are given.

Fat deposition.

From a comparison of the weights of retroperineal and testicular fat in Table III, it is possible to say that in five of the groups of cryptorchid rats, of litters nos. 5, 7, 8 and 23, there is a lack of agreement in the changes obtained between testicular and retroperineal fats, namely that, whilst the testicular fat, as compared with that of the normal control rats, was increased, the retroperineal fat was decreased or showed no change, or *vice versa*. This indicates that, whilst it is only in the majority of cases that the increase, or decrease, in fat deposition is evenly distributed between the retroperineal and testicular regions, in some rats fat is deposited in one or other of these regions preferentially. It is therefore necessary, in animals with normal testes, to use the sum of the weights of the testicular and retroperineal fats to obtain an indication of the deposition of fat in the body. However, when a sufficiently large number of animals is used for the conclusions, the average figures give similar results in both cases. A comparison of the average figures of Table III shows that, on the average, the weights of both the retroperineal and testicular fats were increased in cryptorchid animals, as compared with those of normal control animals. The amount of fat in castrated animals was also increased and in all cases to a greater degree than in cryptorchid animals. For further conclusions based on the total intra-abdominal fat and on a larger number of animals we refer to the previous paper [Korenchevsky, 1930].

Nitrogen content.

In Table II is given the percentage content of nitrogen in each dry hind leg of each rat. The average for each group of each litter and a general average for all the rats of the group are also given. It can be seen, by comparing the nitrogen content of the two legs of the same rat, that in the 42 animals investigated, the fluctuations, from the average for the two legs of the same rat, was about or less than $\pm$ 0·5 % in 21 rats, from $\pm$ 0·5 to $\pm$ 1 % in 10 rats, from $\pm$ 1·1 % to $\pm$ 1·75 % in 7 rats and $\pm$ 2 to $\pm$ 3·2 % in 4 rats.

These results indicate that the method employed was quite satisfactory, giving data sufficiently accurate for the comparison of the different animals, with a probable error not greater than 1·8 % in most cases and in no case investigated more than 3·2 %.

On a general average, it was found that the legs of one-sided cryptorchids contained 1·6 %, of both-sided cryptorchids 3·5 % and of castrates 8·4 % less nitrogen than the legs of their normal controls. Consequently it is necessary to note that the castrated rats were more obese than the cryptorchids and both of them were more obese than the normal rats. We consider therefore that we are justified in explaining this small decrease in the nitrogen content in the same way as we did for Lüthje's dogs (p. 955); that it is unlikely that it can indicate a decrease in the amount of nitrogenous substances in the bodies of cryptorchid and castrated rats; that the decrease is apparent and is due to

the increased proportion of fat in the cryptorchid and castrated animals. This is in agreement with the cases where the nitrogen content was even slightly increased, as was the case in the one-sided cryptorchids of groups 5, 7 and 9 and in the both-sided cryptorchids of group 9. In all these groups the amount of solids (Table IV) was less, in spite of the increased amount of fat in most of the groups. Therefore an even higher nitrogen content is suggested in these cases.

Table IV. *The average percentage content of solids and water in groups of normal, cryptorchid and castrated rats.*

No. of litter	Normal rats		One-sided cryptorchids		Both-sided cryptorchids		Castrates	
	Solids	Water	Solids	Water	Solids	Water	Solids	Water
5	38·1	62·0	37·4	62·6	38·9	61·1	40·8	59·2
6	39·1	60·9	40·2	59·8	41·4	58·7	—	—
7	38·9	61·1	37·7	62·3	39·6	60·4	—	—
8	39·4	60·6	39·7	60·3	41·7	58·3	41·8	58·2
9	40·6	59·5	39·8	60·2	40·0	60·0	40·2	59·8
22	39·6	60·5	—	—	40·4	59·6	39·8	60·2
23	39·1	60·9	—	—	39·7	60·3	—	—
24	37·1	63·0	—	—	40·1	59·9	—	—
25	39·2	60·8	—	—	40·1	59·9	—	—
Average	39·0	61·0	39·0	61·0	40·2	59·8	40·7	59·4

Solids and water content.

From Table IV it is clear that the solids content of the legs was slightly higher, and the water content slightly less, in both-sided cryptorchids and in castrates, than in the normal control rats. On the average, there was no change in one-sided cryptorchids.

Although this increase in the content of solids was only small (by 3·1 % in cryptorchids and 4·4 % in castrates) nevertheless its occurrence in nearly all the groups investigated indicates that it is not of an accidental character. The increased deposition of fat is probably only partly responsible for this increase, since in one-sided cryptorchids the degree of obesity was about the same as in both-sided cryptorchids, but the increase in solids was absent in the former group.

Taking into consideration the increased deposition of nitrogen in most castrated animals [Korenchevsky, 1925], we suggest the same explanation as we have given for Lüthje's results, namely, that in castrated and both-sided cryptorchid animals an increase in the content of solids is to be explained by an increased deposition of fat and probably also of nitrogenous substances in the body.

Summary.

1. A method was used for the investigation of the chemical composition of small animals, which gives approximate but accurate data.

2. This method was applied for ascertaining the content of water, solids, nitrogen and fat in castrated and one-sided and both-sided cryptorchid male

rats, as compared with that of normal litter mate animals. Altogether 46 rats were investigated.

3. The amount of intra-abdominal fat was increased in both cryptorchid and castrated animals, but to a greater degree in the latter.

4. The nitrogen content was slightly decreased in both-sided cryptorchid and in castrated rats.

5. This decrease, however, is chiefly due to the increased proportion of fat in the bodies of cryptorchid and castrated rats, and therefore is not due to the impoverishment of the bodies of these animals in nitrogenous substances.

6. A slight increase in the content of solid matter was found in both-sided cryptorchid and in castrated rats. This increase can be explained by an increase in the deposition of fat and probably also of nitrogenous substances.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We also wish to express our sincere gratitude to Sir Charles Martin for his continuous support and criticism of this work.

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CVII. AN IMPROVED DESIGN OF THE VAN SLYKE APPARATUS FOR THE ESTIMATION OF AMINO-NITROGEN.

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An extended use of the apparatus described by Van Slyke [1912, 1914, 1915] for the quantitative determination of amino-nitrogen has revealed several drawbacks.

There are three points in the glass system at which pieces of thick-walled glass capillary tubing are joined by rubber tubing. The undesirability of rubber connections in systems in which gas volumes are measured needs no emphasis. In the case of the Van Slyke apparatus the abolition of the rubber connections is the more desirable in that shaking of the deaminising chamber and Hempel pipette subjects the rubber to periodic stretchings which must diminish its impermeability to gases. Where deamination takes 1 hour, as it does in the case of lysine, histidine, etc. [Plimmer, 1924], this disadvantage may be serious. It was found necessary always to apply glycerol to the surfaces of contact of glass and rubber and tightly to wire the rubber to the glass.

If thick-walled rubber tubing is used in order to lessen the danger of loss of gas at the joints, shaking subjects the thick-walled glass capillary tubing to strain, on account of the elasticity of the rubber. In use, breaks were frequently found to occur.

These considerations prompted the design of a new form of the apparatus in which the disadvantages referred to were eliminated.

The gas system is made in one piece and mounted without strains on a stout, upright board, having apertures cut in it where necessary. Fig. 1 shows a front view of the apparatus. *A* represents an end elevation of the deaminising bulb which is of the original Van Slyke design. The deaminising bulb is clipped to a wooden block, which is screwed to the board and is not indicated in the figure. The thick-walled capillary tubing as well as the two-way tap *B* are of larger size than usual in order to impart greater strength to the apparatus.

The gas burette is of 10 cc. capacity, graduated in its upper part in 0·01 cc., the bore being as large as conveniently possible. The bulb at its lower end holds 40 cc. A bulb of this size was found necessary when hexone bases were analysed,

since at the end of the hour necessary for deamination considerably more than 20 cc. of gases have been evolved.

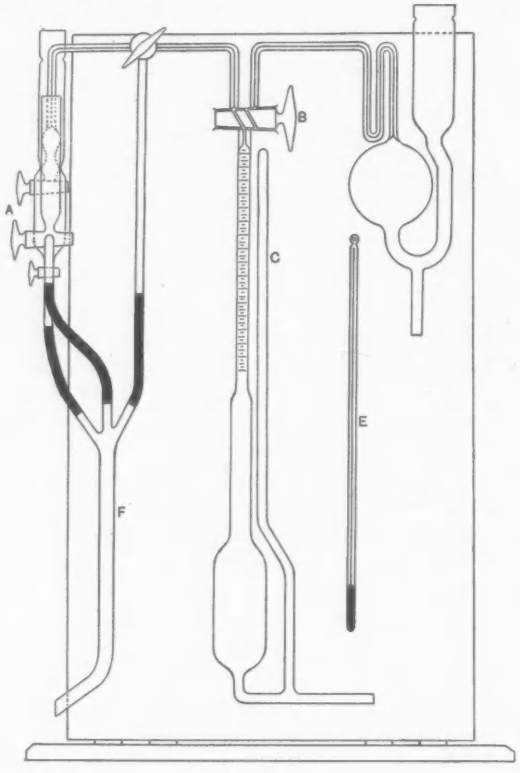

Fig. 1.

The levelling manometer *C* provides greater ease and accuracy in adjusting gas volumes to atmospheric pressure. It is bent over at right angles at the top and fitted with a tap *D*, shown in Fig. 2, so that the manometer can be closed when the manipulations necessitate it. Its diameter should be large enough for the capillary rise of water to be negligible.

The absorption pipette has been slightly modified from the original, the spherical bulb being of 100 cc. capacity, while the cylindrical vessel holds 60 cc.

A thermometer *E* and waste pipe *F* are also fastened to the board, the latter communicating by rubber connections with the three outlets of the deaminising bulb.

The upright board is fastened by strong hinges at the bottom to a base-board and can be oscillated about the hinges by means of a crank attached to the board by the brass coupling *G*. The crank is actuated by the wheel *H*

driven by an electric motor *J*, a considerable variation in the speed of shaking being afforded by a suitable rheostat. The whole apparatus is firmly screwed to the bench.

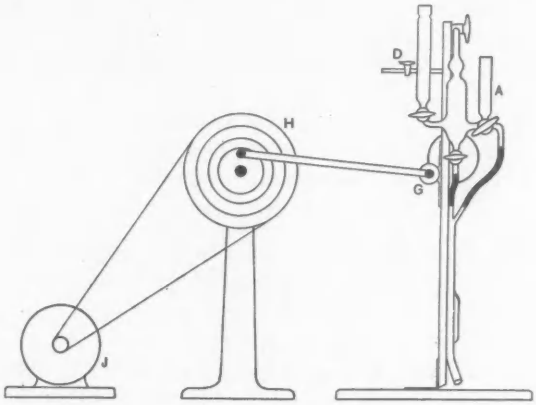

Fig. 2.

The levelling reservoir is placed in a cradle which can be slowly raised or lowered by means of an endless screw.

With this apparatus it was found possible to estimate amino-nitrogen to 0·01 mg. A standard solution of twice crystallised copper aminoacetate was prepared and the quantity of amino-nitrogen in 2 cc. determined, the solution being run in from a calibrated pipette and washed in with 2 cc. water.

Barometer mm. mercury	Temp.	cc. nitrogen evolved	mg. amino-nitrogen
765	18·8°	1·98	1·14 (1)
765	19·0	1·99	1·14 (6)
767	20·0	1·96	1·12 (7)
767	21·0	2·00	1·14 (4)

Theoretical value 1·13 mg.

In 1915, Van Slyke described a modification of his original apparatus in which, it was claimed, volumes of nitrogen could be measured to 0·001 cc. by means of a burette graduated in 0·01 cc. with the graduations about 1 mm. apart.

It seems doubtful whether this degree of accuracy can generally be attained without special precautions to ensure the constancy of the temperature of the gas burette and also to ensure that there is no temperature lag between the latter and the thermometer. A temperature variation of 0·5° will make an appreciable difference in the third decimal place of milligrams of nitrogen.

Further, it seems inadvisable to use a gas burette of so narrow a bore as that specified by Van Slyke, since in such a tube the capillary rise of water will be appreciable. Hence the gaseous volumes will be read under a slightly different pressure from that recorded by the barometer.

During the manipulations it is almost impossible to avoid contamination of the water in the upper part of the burette with acetic acid, sodium nitrite, potassium permanganate and sodium hydroxide, and it is certain that such capillary active substances will appreciably affect the surface tension of the liquid over which the gas is measured and hence change the capillary rise. The pressure of water vapour in the burette will also be changed by the presence of these impurities.

Though the magnitude of these effects is not accurately known, consideration of them makes it evident that, until they have been shown to be negligible, volumes of nitrogen under these conditions can only be given with certainty to 0·01 cc.

I should like to acknowledge the facilities afforded by the Physiology Department of the University of Manchester and to express my thanks to Prof. H. S. Raper and Mr A. D. Ritchie for kind advice.

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CVIII. A SENSITIVE REACTION FOR CYSTEINE.

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Sullivan [1926] found that cystine reduced with sodium cyanide and sodium sulphite gives a colour with sodium β-naphthoquinone-4-sulphonate. This substance is used by Folin [1922] for the estimation of amino-acids and so would appear not to be specific for cysteine. According to Hunter and Eagles [1927], however, the colour given by other amino-acids is not the same as that given by cysteine. The importance of cysteine in biological chemistry and the labour involved in the preparation of the naphthoquinone reagent led us to investigate the possibility of finding a more simple and specific test. We found that cysteine hydrochloride reacts readily with dimethyl-*p*-phenylenediamine hydrochloride when heated in presence of a small quantity of $FeCl_3$ giving a deep blue colour. The colour is stable. It resembles that of methylene blue but does not match it exactly. Like the latter it can be precipitated in presence of $ZnCl_2$ and NaCl at high concentrations. The cysteine employed by us was supplied by the British Drug Houses. We also used other samples prepared from a sample of Merck's cystine by reduction with zinc in hydrochloric acid solution. The solution of cysteine hydrochloride was made by dissolving 0·04 g. in 100 cc. of water, and the solution of dimethyl-*p*-phenylenediamine hydrochloride by dissolving 0·2 g. in 100 cc. of water. It is preferable though not absolutely necessary to use a freshly prepared solution of the latter, as on standing for some time it develops a purple colour. If, however, the purple colour is not too pronounced the addition of the cysteine solution usually renders it colourless again. The concentration of the $FeCl_3$ solution was 5 %. In testing for very small quantities of cysteine, however, it is preferable to use a more dilute solution of $FeCl_3$. The most suitable proportions in which the reagents should be mixed are as follows: to 0·5 cc. of the *p*-phenylenediamine reagent should be added 1 cc. of the cysteine solution followed by one drop of the $FeCl_3$ solution. On heating the mixture almost to boiling point and allowing it to stand the deep blue colour is produced. The mixture of cysteine and *p*-phenylenediamine reagent may first be heated together and the drop of $FeCl_3$ then added to the hot solution when the blue colour develops as before. The test is sensitive with 0·05 mg. of cysteine hydrochloride.

Under similar conditions soluble sulphides do not give methylene blue but require a much more acid medium. The methylene blue reaction for sulphides is given in the cold.

Cystine under the conditions above mentioned gives no colour with these reagents but of course reacts on reduction. Thiolactic and thioglycollic acids do not react with these reagents. Using yeast extracts as a source of glutathione we found that neither oxidised nor reduced glutathione reacted with our reagents. On hydrolysis, extracts of fresh yeast give a positive reaction indicating the presence of cysteine in the decomposition products of glutathione. We hope soon to be able to publish a method, based on this reaction, for the estimation of cysteine and glutathione in tissue extracts.

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CIX. VITAMIN C AND THE RAT'S DIET.

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THE rôle of the antiscorbutic vitamin in the rat's diet is a question upon which there appear to be different views. On the whole nutrition workers in America omit orange and lemon juice from synthetic diets, while in England it is usual to add small quantities. Simmonds [1924] states that "the rat is apparently immune to scurvy and does not require this vitamin in its diet," and Mendel [1923], in discussing the essential factors of a diet, says that the rat does not seem to suffer from a lack of antiscorbutic foods. Parsons [1920] found that the livers of rats fed for long periods on a typical scorbutic diet were rich in the antiscorbutic substance and suggests that the rat needs this factor for its normal metabolism, but is capable of synthesising it. Many American workers do not even mention vitamin C in discussing the composition of their rats' diets, but tacitly assume that this food factor is not needed by the animal. For instance, Anderson and Mendel [1928] supplement their experimental diets by daily doses of yeast and cod-liver oil to provide "an adequate intake of the indispensable vitamins." Sure [1924, 1925] uses cod-liver oil and Harris uses yeast-vitamin powder, but no orange or lemon juice either with his skimmed milk or pure synthetic diets, and Elmslie and Steenbock [1929] give yeast as a source of the vitamin B complex and cod-liver oil to supply vitamins A and D, but no vitamin C. Kennedy and Palmer [1922] also use no vitamin C in their basal diet and express the view that very few workers regard vitamin C as a necessary constituent in the diet of growing rats. Evans and Bishop [1923] used no source of vitamin C in their synthetic diet, but in trying to cure sterility they gave orange juice to some rats and fresh green lettuce to others; they found the latter to be a most efficient cure, but apparently noted no effects due to addition of the orange juice. On the other hand, Harden and Zilva [1918] consider that rats grow better when the antiscorbutic factor is present in the diet, and their growth curves exhibit an appreciable improvement. Drummond [1919] agrees with the work of Harden and Zilva and states that "the rat requires the antiscorbutic factor in order to achieve a normal development." Kennedy [1926] says that although the rat appears to thrive without vitamin C he added concentrated orange juice in case it might be essential for reproduction. The experiments of Bacharach [1925]

showed that no disadvantage attended the omission of lemon juice from the rat's diet during the experimental period involved in testing for vitamin B, and other workers [Coward, Key, Morgan and Cambden, 1929; Drummond and Marrian, 1926; Reader, 1929; Willimot, 1926] now omit lemon juice from a vitamin B-free diet. Coward [1929, 1930] and Coward, Key and Morgan [1929] also omit vitamin C from their vitamin A-free diet.

The amount of orange or lemon juice added to synthetic diets varies a little, but as a rule 5–6 cc. per 90–100 g. dry solid is given by nutrition workers in England [Boas, 1926; Chick, 1926; Chick and Roscoe, 1926; Luce and MacLean, 1925; Reader and Drummond, 1926].

The diets used by Harden and Zilva [1918] contained much larger amounts of lemon juice, *i.e.* 40 instead of 5 cc. Since the growth of rats fed on synthetic diets is far below that obtained on a good mixed diet of kitchen scraps, it is worth while to try any factor which might produce an improvement. Therefore experiments were made in which rats were given large amounts of lemon juice in the synthetic diet to see if it would be an advantage to incorporate more than the usual 5 cc. of orange or lemon juice in the basal diet.

Experimental.

(*a*) *Animals and method.*

Piebald rats bred in the laboratory were used in this work. Twelve animals, 6 ♀ and 6 ♂ (except in the trial experiment with lemon juice, where owing to a shortage of rats only 5 ♀ and 4 ♂ were available) were used for each experiment and were kept in a large metal cage with a screen, so that they had no access to excreta. The pregnant does were removed to smaller wooden cages a few days before the birth of a litter. The rats were weighed daily for the first 4 weeks, after which the weighing was omitted on Saturdays and Sundays. After the birth of the first litter, when the rate of growth is slower, the males were weighed only once a week, though the mother and her young were weighed daily.

(*b*) *Diets.*

Two basal diets were used, one containing caseinogen and the other edestin, as it was thought advisable to use two different proteins in case one produced greater growth than the other, as a result of which the effect of vitamin C might be more marked. The composition of the basal diets was as follows:

Diet AF	Diet AF Ed
Caseinogen 20 g.	Edestin 20 g.
Potato starch 56 g.	Potato starch 56 g.
Mineral salts 8 g.	Mineral salts 8 g. [Hartwell, 1922].
Butter 16 g.	Butter 16 g.
Marmite 15 g.	Marmite 15 g.

The diet was prepared by the "cooked" method described in a previous paper [Hartwell, 1925]. Lemon juice strained through muslin and the green leaves of cabbage finely minced were used as sources of vitamin C, and both were well beaten into the diet when it had cooled. Food and distilled water were given *ad lib.*, but special feeding dishes were used in order to prevent scattering of the food and thus to obtain an approximate intake per cage.

(*c*) *Experiments.*

1. *Diet AF.* This experiment was made in duplicate in order to see what differences in growth might occur with the same diet.

2. *Diet AF + lemon juice.* The proportion of lemon juice added was 40 cc. to every 84 g. of dry diet (*i.e.* caseinogen, starch and mineral salts). A trial experiment was made using 5 ♀ and 4 ♂ to see if the animals could tolerate this amount of lemon juice, or if such a diet would be too acid and therefore cause intestinal disturbances. The young rats developed diarrhoea after 3–4 days on the diet, but after 10 days the faeces were quite normal. Temporary digestive disturbances often occur when weanling rats are put on synthetic diets, but usually the young animals appear to be able to adjust themselves to their new diet in a week or 10 days. In a second experiment 6 ♀ and 6 ♂ were used, and the experiment was continued for 15 weeks.

3. *Diet AF + cabbage.* 72 g. minced green cabbage leaves were added to the basal diet instead of 40 cc. of lemon juice. For the first 2 weeks minced lettuce was given, but this proved so expensive that green cabbage was used instead.

4. *Diet AF Ed.* This diet was similar to diet AF, but pure edestin[1] was used instead of caseinogen.

5. *Diet AF Ed + lemon juice.* 40 cc. lemon juice (strained) were added to 84 g. of the dry diet as in the experiment AF + lemon juice.

Results and discussion.

(1) *Growth.* In all experiments the rats grew well, though the rate of growth was not equal to that of the control animals fed on a mixed diet of kitchen scraps + bread and milk. The addition of lemon juice appeared to have no appreciable effect on the rate of growth. Fig. 1 shows the average growth curves of the males and of the females up to the time they became pregnant. The curves show that there is no greater difference in weight between the rats receiving lemon juice and those getting no vitamin C than there is between the two curves obtained at different times on diet AF alone. In the trial experiment with lemon juice the growth is just better than when no lemon juice was included in the diet. The growth curves when cabbage was used as a source of vitamin C show similar results. When edestin was given

[1] I am indebted to Messrs E. T. Pearson & Co., Ltd., for the pure edestin used in these experiments.

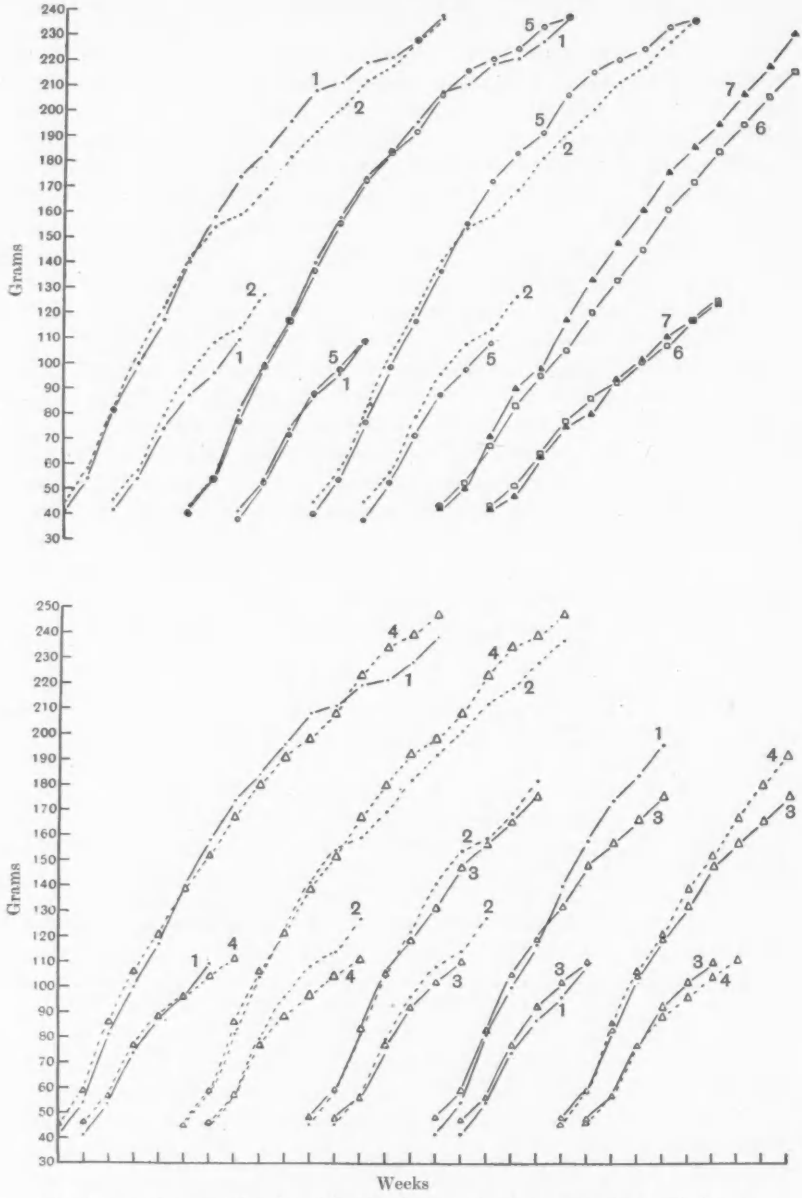

Fig. 1. Growth curves of male and female (shorter owing to pregnancy) rats. Each curve is the average for six rats, except diet AF + lemon juice, Exp. 1.

1. • ——— • Diet AF. Exp. 1.
2. • - - - - - • Diet AF. Exp. 2.
3. △ —— △ Diet AF + lemon juice. Exp. 1.
4. △ - - - △ Diet AF + lemon juice. Exp. 2.
5. ○ —— ○ Diet AF + cabbage.
6. □ —— □ Diet AF Ed.
7. ▲ —— ▲ Diet AF Ed + lemon juice.

instead of caseinogen the rate of growth appeared to be just better when lemon juice was added, but this improvement is very slight, the actual difference in weight being no greater than that obtained in the two separate experiments on diet AF.

(2) *General condition.* Although the animals grew well, their general condition was not up to standard. None of them was quite so firm to feel as the stock animals and practically every rat exhibited loss of fur, though the coat grew again without any alteration in the diet. The lemon juice caused no improvement in the caseinogen diet, but with edestin the fur did not come out quite so badly when lemon juice was added.

(3) *Reproduction.* On the whole reproduction was very poor and therefore the experiments were not continued after the first litters were born. Both with presence and absence of lemon juice the young were frequently born dead or died within a few days of birth, only a few were reared and the numbers per litter were low compared with stock animals.

From these experiments, therefore, it appears that the addition of lemon juice to the basal diet produces no improvement in breeding, but green cabbage leaves have a beneficial effect. Hence it seems that vitamin C itself is not the factor to which the improvement is due, and it is obvious that the raw cabbage contained some food factor of importance in breeding, which was not present in the lemon juice. It seems unlikely that the diets used were at all deficient in vitamin E, for Sure [1927] finds that 10 % of butter-fat in a synthetic ration assures fertility and successful lactation in the rat, and the diets described in this paper contained 16 % of butter. Mattill and Clayton [1926] consider it probable that milk-fat contains more vitamin E than has been estimated, though Evans and Bishop [1923] find that large quantities of this fat are required to cure sterility. However, the curative dose may be greater than the preventive ration. No information can be found as to the relative amounts of vitamin E in lemon juice and cabbage, therefore the reason for the improvement produced by cabbage leaves must await further research. It is interesting, however, to note that no improvement in growth was obtained by the addition of cabbage as compared with the lemon juice, affording further evidence that the dietary requirements vary with different periods of existence.

The experiments described here do not support the view of Harden and Zilva [1918] that the addition of the antiscorbutic vitamin to the rat's diet produces better growth. These workers obtained distinctly better growth when they added lemon juice with the citric acid removed, but as suggested by Palmer and Kennedy [1927] the lemon juice may have contained vitamin A or B, or both, and therefore the favourable result may not have been exclusively due to vitamin C. Since the experiments of Harden and Zilva much work has been done on growth with various diets and it is probable that there are several possible explanations of their results. For instance, their AB diet contained different amounts of the various constituents as compared with their ABC diet—thus in the AB diet there were 2·5 cc. olive oil, while the ABC diet had only 0·25 cc.

More recent work has shown that the actual proportion of constituents of a diet is an important factor and this in itself may account for the different rates of growth. It is also possible that the modified technique adopted now, *i.e.* keeping animals on screens away from their excreta, will require an alteration in the amounts of the different food factors required. In previous work [Hartwell, 1926] good growth was obtained with 2 g. marmite per 100 g. solid, but more recent work when rats were kept on screens has shown this figure to be too low. With their excreta accessible animals make up deficiencies in their diets from this source, but a more generous supply in their food may well produce better growth.

SUMMARY.

The addition of the antiscorbutic vitamin to a synthetic diet produced no improvement in the rate of growth or reproduction of the rat.

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CX. STUDIES ON HEMICELLULOSES.

II. THE HEMICELLULOSES OF MAIZE COBS.

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Introduction.

Maize cobs have long been recognised as a source of xylose, which may be obtained from them in a pure condition and in fairly high yield. More recently, a method for the preparation of xylose from this source has been described by Ling and Nanji [1923], who obtained the sugar in 10–12 % yield.

This xylose arises, at least in part, by the hydrolysis of hemicelluloses present in the cobs. Johnson [1896] investigated the nature of maize cob hemicellulose. After a preliminary treatment with dilute ammonia, the cobs were extracted with sodium hydroxide solution, the hemicellulose being precipitated by addition of alcohol. The product yielded xylose on hydrolysis, other sugars not being detected, while as a result of elementary analysis the formula $(C_5H_8O_4)_n$ was ascribed to it.

Maize cob hemicellulose was thus regarded as a single substance, indeed as a pure xylan.

However, it has been indicated in a previous paper dealing with the hemicelluloses of wheat bran [Norris and Preece, 1930] that in some cases at least it may be possible to separate the hemicellulose from a given source into a number of fractions, having constitutions which are similar but, nevertheless, distinct. Moreover, it has been shown that uronic acid residues are widespread in hemicelluloses [O'Dwyer, 1926; Norman, 1929].

It was felt, therefore, that the method of separation successfully applied in the case of the hemicelluloses of wheat bran might to advantage be attempted with the hemicelluloses of maize cobs, with the added object of detecting the presence of constituents other than xylan. In a substance of such complexity as a hemicellulose, the substitution of one or more residues of xylose by residues of other sugars, or sugar derivatives, would but slightly alter its elementary composition. Thus, the analysis of maize cob hemicellulose might still approximate very closely to that for a pure xylan, even though xylose were not the only sugar obtainable from it on hydrolysis.

Experimental.

Preparation and purification of the hemicelluloses.

As a preliminary to chemical treatment, the cobs were mechanically reduced to a coarse sawdust. The preparation of the hemicelluloses was carried out in exactly the same way as has been described for the preparation of the hemicelluloses of wheat bran [Norris and Preece, 1930]. The method there described again gave three fractions, designated hemicelluloses A, B and C.

Furfuraldehyde determinations were carried out on the products, and the results obtained (calculated on the dry, ash-free material) are quoted in Table I where the yields from 400 g. of the ground cobs are also given.

Table I.

Fraction	Yield of crude product from 400 g. of cobs (g.)	Furfuraldehyde %
A	32	60·70
B	24	57·82
C	6	50·77

Hemicellulose A was purified by dissolving in 4 % sodium hydroxide solution, filtering through paper pulp, and precipitating with Fehling's solution as the copper complex. This was decomposed with dilute hydrochloric acid, and the hemicellulose precipitated and washed with acetone.

An attempt was made to separate hemicellulose B into two fractions analogous to hemicelluloses B 1 and B 2 of wheat bran. However, the whole of this fraction was directly precipitated from alkaline solution by Fehling's solution; consequently this product is described as hemicellulose B 1.

On the other hand, hemicellulose C was split up by copper treatment, a portion—hemicellulose C 1—being directly precipitated by Fehling's solution, while hemicellulose C 2 was precipitated from the filtrate from this on addition of half a volume of acetone. Both fractions were further purified by a repetition of the treatment.

Thus, the hemicelluloses of maize cobs, like those of wheat bran, may be separated into four fractions, the two sets having, however, somewhat different characteristics. Thus, while under the present system of nomenclature the wheat bran-hemicelluloses are designated A, B 1, B 2 and C 2, those of maize cobs are A, B 1, C 1 and C 2.

General properties of the hemicelluloses.

The four hemicelluloses are white, amorphous, non-reducing powders. Fraction A is insoluble in hot water; B 1 swells up in cold water, and is completely dissolved on boiling; C 1 is partially dissolved by cold water, complete solution being obtained on boiling; C 2 shows gum-like swelling in cold water; hot water dissolves it entirely. None of these hemicelluloses gives an iodine coloration.

During the estimation of furfuraldehyde, it was found that the phloroglucide precipitates obtained from hemicelluloses B, C 1 and C 2 contained appreciable amounts of a substance soluble in 95 % alcohol to a red-brown solution, indicating the presence of methylpentosan in these hemicelluloses. Consequently, determinations of methylpentosan in presence of pentosan were carried out according to the method of Tollens and Ellett [see Browne, 1912]. The percentages of methylpentosan found and the percentage yields of furfuraldehyde are given in Table II. Carbon dioxide numbers were also obtained by the method of Nanji, Paton, and Ling [1925]. The rotatory powers of A, B 1 and C 1 in 4 % sodium hydroxide solution were also determined.

All the results given in this table are calculated on the dry, ash-free hemicelluloses, and in each case the figures quoted are the mean for a number of closely agreeing determinations.

Table II.

Hemicellulose	A	B 1	C 1	C 2
Yield from cobs %	8·0	6·0	1·0	0·5
Methylpentosan %	—	7·01	7·42	8·91
Furfuraldehyde %	61·49	57·31	54·57	43·90
Carbon dioxide %	0·94	1·30	1·85	1·39
Uronic anhydride %	3·76	5·20	7·40	5·56
Rotatory power $[\alpha]_D^{20°}$	− 96°	− 108°	− 90°	—

Since hemicellulose C, before separation into its constituents, was found to give a furfuraldehyde yield of 50·77 %, its components C 1 and C 2 are present in the ratio of two parts of the former to one of the latter.

Hydrolysis.

Hemicellulose A. Some 5 g. of the hemicellulose were hydrolysed by heating under reflux for 4 hours with 250 cc. of 1 % sulphuric acid. The sulphuric acid was then neutralised by the addition of the requisite volume of saturated barium hydroxide solution, and the precipitated barium sulphate filtered off. The filtrate was evaporated *in vacuo* just to dryness, and the residue extracted twice with 50 cc. portions of absolute alcohol. The alcoholic extracts after filtering were united, the alcohol removed by evaporation, and drying completed *in vacuo*. The residue insoluble in absolute alcohol was twice extracted with 50 cc. portions of boiling 80 % alcohol, the united extracts being taken to dryness as before. The amount of residue after 80 % alcohol extraction was extremely small, and was neglected.

The bulk of the hydrolysis product was found to have passed into the absolute alcohol. The sugar of this fraction gave $[\alpha]_D^{20°} = + 18°$, and melted after recrystallisation at 139°.

From a portion of this sugar an osazone was prepared. The product was pure xylosazone, having the characteristic crystalline appearance and melting after recrystallisation at 160°. The presence of xylose was confirmed by the preparation of the characteristic cadmium xylonobromide.

From the sugar soluble in 80 % alcohol a mixture of osazones was obtained. One, insoluble in hot water, had a crystalline appearance similar to that observed in the case of insoluble phenylhydrazine derivatives obtained from the hydrolysis liquors of certain of the hemicelluloses of wheat bran [Norris and Preece, 1930]. After purification it melted sharply at 160°. In all probability, this product is a derivative of glycuronic acid. The second osazone was that of xylose, and the presence of this sugar was confirmed by the preparation of cadmium xylonobromide.

Sugars other than xylose could not be detected in either of the two fractions.

In a second hydrolysis, a weight equivalent to 1·549 g. of dry, ash-free hemicellulose A was treated with 1 % sulphuric acid in the usual way, the sulphuric acid neutralised with the requisite volume of barium hydroxide solution, and the barium sulphate removed by filtration. The precipitate was carefully washed with water, and the filtrate and washings combined. The liquid still remained acid to phenolphthalein, and a further amount of barium hydroxide was added to bring the reaction to the neutral point. After evaporation to dryness *in vacuo*, the sugar was extracted several times with small quantities of boiling absolute alcohol, and the extracts filtered hot, and combined.

From the combined extracts there was recovered 1·45 g. of xylose, giving for $c = 3{\cdot}362$ $[\alpha]_D^{20^\circ} = +19{\cdot}5^\circ$. It is shown below that hemicellulose A contains 94·36 % of xylan, so that the present amount of xylose obtained represents some 88 % of the theoretical yield.

Hemicellulose B 1. The neutral hydrolysis liquor from hemicellulose B 1 was taken to dryness *in vacuo* and extracted successively with absolute alcohol and with 80 % alcohol. The bulk of the sugar was again found to pass into the absolute alcohol.

The sugar soluble in absolute alcohol gave $[\alpha]_D^{20^\circ} = +18^\circ$. An osazone prepared from it proved to be xylosazone, melting after recrystallisation at 158°. Cadmium xylonobromide was also prepared.

The product soluble in 80 % alcohol showed $[\alpha]_D^{20^\circ} = +19^\circ$. This also was xylose, yielding xylosazone melting at 158°, and yielding cadmium xylonobromide.

Hydrolysis products other than xylose could not be detected in either fraction, nor was a phenylhydrazine derivative insoluble in hot water obtained from either.

A hydrolysis on quantitative lines was carried out in a manner similar to that employed in the case of the second hydrolysis of hemicellulose A described above.

1·72 g. of hemicellulose B 1 yielded 1·58 g. of xylose, showing for $c = 2{\cdot}31$ $[\alpha]_D^{20^\circ} = +19^\circ$, and melting at 140–141°. This represents a yield of xylose approximating to 92 % of the theoretical, since, as is shown below, hemicellulose B 1 contains 87·60 % of xylan.

Hemicelluloses C 1 and C 2. Owing to the small amounts of these products available for examination, only preliminary investigations of the nature of the sugars arising on hydrolysis could be attempted. However, these sufficed to indicate with certainty the nature of the pentoses produced.

To the hydrolysis liquor obtained from 1 g. of hemicellulose C 1 saturated barium hydroxide solution was added until the liquid remained just acid to phenolphthalein. The filtrate from barium sulphate was concentrated to a bulk of 50 cc. and divided into several portions for examination.

An osazone was prepared. The product was mainly pentosazone, soluble in hot water. This had the crystalline form of xylosazone and melted after several recrystallisations at 154°, a somewhat low temperature. The small amount of derivative insoluble in hot water had the crystalline form associated with the phenylhydrazine derivative of glycuronic acid. It was not further examined.

The presence of xylose was confirmed by the preparation from a second portion of hydrolysis liquor of the characteristic cadmium xylonobromide. Arabinose and hexoses were absent.

The hydrolysis liquor from 1 g. of C 2 was treated similarly to the above. The osazone obtained was again a mixture. The small amount insoluble in hot water was the glycuronic acid derivative, melting after recrystallisation from dilute alcohol at 155–156°. The hot water-soluble osazone resembled in crystalline appearance the osazone of arabinose; it melted at 158°.

By treating a portion of the hydrolysis liquor in the usual way with diphenylhydrazine, a copious precipitate of colourless crystalline arabinose diphenylhydrazone was obtained on standing. After recrystallisation from 50 % alcohol this gave a melting point of 200°. The presence of arabinose amongst the products of hydrolysis of hemicellulose C 2 was thus confirmed. Xylose and hexoses were absent.

In the case of none of the hemicelluloses was it possible to identify the methylpentosan constituent. Further, the very small amounts of uronic acid residues present in all cases made their absolute characterisation impossible, though, as has been shown, glycuronic acid appears to be present in at least three cases.

Discussion.

It has been shown by Cunningham and Dorée [1914] and other workers, that ω-hydroxymethylfurfuraldehyde gives with phloroglucinol a precipitate which is soluble in alcohol. However, it has been shown that the hemicelluloses of the present research contain no hexosan, and it thus seems legitimate to regard the alcohol-soluble portion of the phloroglucide precipitate as due solely to methylpentosans.

From the results of the hydrolyses, and from the analytical data quoted in Table II, it is possible to calculate with fair accuracy the relative proportions of xylan, araban, methylpentosan, and uronic anhydride present in the four fractions. Use is made of the following relations:

100 g. uronic anhydride yield 16·7 g. furfuraldehyde.
100 g. xylan yield 64·5 g. furfuraldehyde.
100 g. araban yield 53·5 g. furfuraldehyde.

The calculated values are given in Table III.

Table III.

Hemicellulose	A	B 1	C 1	C 2
Total furfuraldehyde %	61·49	57·31	54·57	43·90
Furfuraldehyde from uronic anhydride %	0·63	0·87	1·23	0·93
Furfuraldehyde from pentosan % ...	60·86	56·44	53·34	42·97
Xylan %	94·36	87·50	82·70	—
Araban %	—	—	—	80·32
Methylpentosan %	—	7·01	7·42	8·91
Uronic anhydride %	3·76	5·20	7·40	5·56
Total percentage accounted for	98·12	99·71	97·52	94·79

Thus, it has been possible in all cases to account satisfactorily for practically 100 % of the constituents. It will be seen that hemicelluloses A, B 1 and C 1 form a series, in which increasing solubility in water is accompanied by simultaneous increases in the amounts of uronic anhydride and methylpentosan present, with corresponding decreases in the xylan percentages. However, the corresponding rotatory powers (Table II) do not show a similar progressive change.

The simultaneous occurrence in some cases of methylpentosan and uronic anhydride is of interest; this might have been expected, however, since these may be regarded as derived from hexosan by reduction and oxidation respectively.

If the above fractions represent individual hemicelluloses, it is apparent that the hemicellulose molecule is one of considerable complexity. Thus, fraction B 1, yielding 57·31 % furfuraldehyde, 7·01 % methylpentosan, and 5·20 % uronic anhydride, would correspond to a molecule of the minimum dimensions

$$(C_5H_8O_4)_{43} \cdot (C_6H_{10}O_4)_3 \cdot (C_6H_8O_6)_2$$

with a molecular weight of approximately 6500. Such a structure would give a furfuraldehyde yield of 57·47 % and would contain 6·8 % and 5·4 % of methylpentosan and uronic anhydride respectively.

The alternative view that the fractions isolated represent aggregates of molecules of similar physical properties has much to commend it, and at present it is manifestly unwise to claim that individual hemicelluloses have been isolated.

Summary.

1. The hemicellulose of maize cobs has been separated into four fractions, none of which corresponds to a pure xylan.

2. Hemicellulose A yields on hydrolysis xylose and a uronic acid; B 1 and C 1 yield varying proportions of xylose, uronic acid, and methylpentose; C 2 gives arabinose, uronic acid, and methylpentose.

3. In all cases the proportion of pentose residues present far exceeds the combined proportions of residues of methylpentose and uronic acid.

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CXI. CAROTENE AND VITAMIN A.

THE TRANSFORMATION OF CAROTENE INTO VITAMIN A AS SHOWN BY A STUDY OF THE ABSORPTION SPECTRA OF RAT-LIVER OILS.

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(*Received June 28th, 1930.*)

MOORE [1929, 1930] has advanced evidence to show that when highly purified carotene is administered to rats it is not stored in the liver in an unchanged condition, but suffers conversion to vitamin A. Among other evidence in favour of this view, preliminary results obtained by the present author in regard to the absorption spectra of the liver oils of rats receiving carotene were quoted. These preliminary experiments have now been confirmed and the results may be described in detail.

EXPERIMENTAL.

The liver oils, extracted by Dr Moore, were forwarded to me in sealed tubes. After weighing, the oils were dissolved in dry ether or chloroform and their absorption spectra determined with a Hilger E 3 all-quartz spectrograph used in conjunction with a Hilger sector photometer with a condensed copper spark as light source.

The numbering of the rats corresponds with that of Moore's paper [1930]. Closely similar absorption curves were obtained for the liver oils of rats receiving similar diets and representative examples are reproduced in Fig. 1.

The results are shown in Table I.

Table I.

No. and sex of rat	Diet	Solvent	Band at 325 $\mu\mu$	Band or inflexion in region of 280 $\mu\mu$
5 ♂	Vitamin A-free	Ether	No band	Inflexion
8 ♀	,,	Chloroform	,,	,,
9 ♂	,,	,,	,,	,,
10 ♀	,,	Ether	,,	,,
16 ♀	Vitamin A-free + 0·75 mg. per	Ether	Good band	Shallow band
17 ♀	day carotene. M.P. 178°	Chloroform	,,	,,
19 ♂	Vitamin A-free + red palm oil	Chloroform	,,	Inflexion
20 ♀	(1·5 g.)	Ether	,,	Shallow band
22 ♂	Vitamin A-free + fresh carrots *ad lib.*	Chloroform	,,	Shallow band

Discussion.

It is seen that in every case the liver oils of rats fed on a diet free from vitamin A gave no band in the region of 325$\mu\mu$, while the liver oils of rats given in addition either crystalline carotene or substances containing carotene showed selective absorption in this region in every case. The absorption spectra shown by the liver oils of the rats treated with carotene were therefore similar

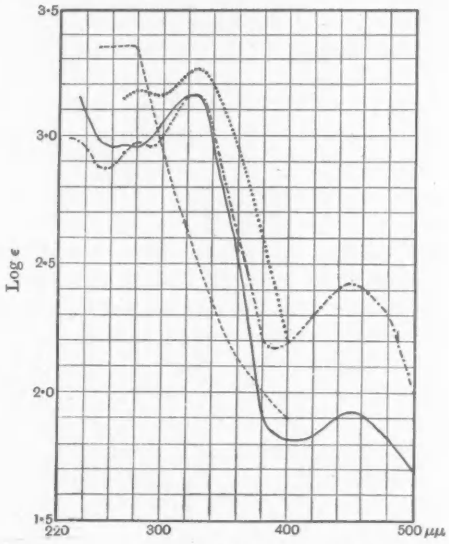

Fig. 1. - - - - - Liver oil of rat no. 10 in chloroform.
. . . . . Liver oil of rat no. 22 in chloroform.
——— Liver oil of rat no. 16 in ether.
— · — Liver oil of rat no. 20 in ether.

ε is defined by $\log I_0/I = \varepsilon cd$, where c is the concentration of the liver oil in g. per cc., d is the cell thickness in centimetres.

to those shown by cod-liver oils in which, as well as in other animals, vitamin A activity has been found to run parallel with a prominent band in the position stated [Takahashi *et al.*, 1925; Morton and Heilbron, 1928; Drummond and Morton, 1929]. Since it has already been shown [Capper, 1930] that this band is absent from the absorption spectrum of carotene (subsequent experiments have also shown that it is absent from the spectrum of red palm oil) it is obvious that the substance responsible (presumably vitamin A) must be synthesised *in vivo* from the carotene.

This result not only affords support to the view that carotene is converted to vitamin A *in vivo* but in a reciprocal manner serves also to strengthen confidence in the reliability of the 325$\mu\mu$ band as an index of the presence of the vitamin. Morton and Heilbron [1928] have shown that this band is always present in sources of vitamin A of animal origin, and that when physiological

activity is destroyed by oxidation the band disappears. From the present work additional evidence may be deduced, since when the vitamin, as characterised by its other attributes, is synthesised in the rat, the band simultaneously makes its appearance.

In addition to the main band at 325 $\mu\mu$ shallow bands or inflexions were noted in the region of 280 $\mu\mu$. Absorption in this position has been repeatedly described by various workers in the case of cod-liver oils [Schlutz and Morse, 1925; Schlutz and Ziegler, 1926; Heilbron, Kamm and Morton, 1927; Woodrow, 1928]. It has been suggested that this band may be due either to the presence of ergosterol or to a decomposition product of vitamin A, but nevertheless the actual significance remains obscure. From the present work it may be noted, without comment, that inflexions were observed even in the case of the liver oils of rats killed during acute vitamin A deficiency.

Summary.

1. The band at 325 $\mu\mu$ generally attributed to vitamin A was absent from the absorption spectra of the liver oils of rats suffering from vitamin A deficiency, but was shown by the liver oils of similar rats which had been subsequently cured by massive doses of carotene.

2. Since the 325 $\mu\mu$ band has already been shown to be absent from the absorption spectrum of carotene, it is obvious that the substance responsible for the exhibition of this band by the liver oils of carotene-treated rats (presumably vitamin A) had been synthesised *in vivo* from the carotene.

3. Additional evidence in favour both of the conversion of carotene to vitamin A and of the reliability of the 325 $\mu\mu$ band as an index of the presence of the vitamin is therefore made available.

My thanks are due to Dr Moore for his continued interest and help.

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CXII. NOTE ON THE POSSIBLE MECHANISM OF DIPHTHERIA TOXOID FORMATION.

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When diphtheria toxin is incubated with formaldehyde the toxic properties disappear [Glenny and Sudmersen, 1921], leaving the antigenic function practically unimpaired [Ramon, 1925, 1928]. In these laboratories toxins have been prepared with an M.L.D. as low as 0·0005 cc. and Lf. of 0·024 cc. and after incubation with 0·4 % formalin the M.L.D. is considerably greater than 5 cc. whilst 90 % of the antigenic activity (as measured by the flocculating power) is retained.

The mechanism of this process is of considerable interest both from the theoretical and practical points of view since toxoid is largely used in the preparation of diphtheria antitoxin and is being employed increasingly in prophylaxis.

Formaldehyde reacts with amino-groups forming methylimino-compounds. It appears possible therefore that the toxoiding process is due to the removal of free amino-groups in the toxin which are responsible for toxicity but play no part in eliciting antibodies or combining with antitoxin.

Kissin and Bronstein [1928] claim that all free NH_2-groups in the toxin molecule are combined during the toxoiding process, but later [1930] modify this view and claim that one-half of the free NH_2-groups disappears when the toxin is treated with 0·4–0·5 % of formalin. They conclude therefore that there are two sets of amino-groups in the toxin molecule. One set is responsible for toxicity and, conveniently, disappears during the toxoiding process; and the other set, responsible for combining with antitoxin and eliciting antibodies, remains unattacked by formaldehyde.

Experiments are described in this communication to test this and other hypotheses which might explain toxoid formation.

Experimental.

Method of toxoid preparation.

Directions for preparing diphtheria toxoid given by various authors are as follows: the toxin is incubated at 36 to 42° with 0·3 to 0·5 % of formalin for periods ranging from 2 weeks to 2 months.

In these laboratories the following standard conditions have been found most suitable.

0·4 % of 40 % formaldehyde is added to the toxin and the mixture is incubated at 38° in a hot room for 4–6 weeks. The loss of antigenic power (as measured by flocculation methods) is of the order of 10 % only, and the toxoid produces no local or general reaction when inoculated subcutaneously in 5 cc. doses into 300 g. guinea-pigs.

To give a typical example: a 5 litre batch of toxin (M.L.D. 0·0005 cc., Lf. 0·024 cc.) was incubated at 38° with 20 cc. of 40 % formaldehyde (A.R.) for 6 weeks. 5 cc. were then inoculated subcutaneously into a 300 g. guinea-pig. No local swelling or other symptom was observed and the animal steadily gained weight. The Lf. was now 0·028 cc. Thus the toxicity had diminished at least 20,000 times, whilst 90 % of the flocculating power was retained.

The following experiment shows the effect of varying the conditions of the toxoiding process.

Original toxin, Lf. 0·09 cc., 5-litre batches used; after 4½ weeks' incubation under the conditions described, subcutaneous inoculations of 5 cc. were made into 300 g. guinea-pigs with the results shown.

Table I. *Effect of amount of formalin added on toxoid formed.*

(4½ weeks' incubation at 38°)

Formalin added %	Effect on guinea-pig: Change in weight after 1 week (g.)	Effect on guinea-pig: Amount of local swelling
0·3	− 5	Large firm
0·4	+ 15	Trace
0·5	+ 25	Practically nil

Table II. *Effect of temperature of incubation on toxoid.*

(0·4 % formalin)

Temp. ° C.	Effect on guinea-pig: Weight after 1 week (g.)	Effect on guinea-pig: Amount of local swelling
36	− 35	Extensive firm
38	+ 15	Trace

Table III. *Effect of* p_H *after addition of formalin.*

(0·4 % formalin at 38°)

p_H when incubation commenced	Effect on guinea-pig: Weight after 1 week (g.)	Effect on guinea-pig: Amount of local swelling
8·0	± 0	Practically nil
7·3	+ 15	Trace

In another experiment one batch of toxin was incubated with 0·4 % of formalin for 6 weeks at 40–41° and another batch at 38°. In this case practically no difference was observed between the effects on guinea-pigs of toxoids prepared at 38° and 40°, but whilst the flocculating titre of that prepared at 38° had dropped some 8 %, that incubated at 40° had fallen nearly 25 %. It is

inadvisable and unnecessary therefore to incubate at temperatures higher than 38°.

Amino-nitrogen of toxins.

Veal or horse-flesh infusion broth containing 2 % of Difco proteose peptone is used for toxin production. The amino-nitrogen of the medium (determined by the Van Slyke and Sørensen methods) varies between 0·065 and 0·1 %. The toxin filtrates prepared by 8–10 days' growth of Park-Williams 8 strain of *C. diphtheriae* have generally a slightly higher content of amino-nitrogen, but lower values than those of the original medium have been observed. The limits observed have been 0·06 % and 0·11 % of amino-nitrogen.

In view of Hartley and Hartley's [1922] observation of the deleterious effect of overheating the medium required for toxin production, comparisons were made of toxin production in broths sterilised by autoclaving and by candle-filtration respectively. Batches of broth were divided into two parts, both being treated in precisely the same way, save that one part was autoclaved and the other filtered through a candle to effect sterility. In each case better toxin production and higher amino-nitrogen content were observed in the batches which had not been autoclaved. No direct relationship, however, could be traced between toxin production and the amino-nitrogen content of the medium.

Table IV. *Effect of method of sterilisation of broth on properties of toxic filtrate.*

	Filtered		Autoclaved	
Batch no.	Flocculation units per 1 cc.	Amino-nitrogen %	Flocculation units per 1 cc.	Amino-nitrogen %
821	42	0·10	18	0·05
824	28	0·09	8	0·06

Action of formaldehyde on amino-compounds.

Amino-groups are determined in the Sørensen method by addition of excess of formaldehyde and titration of the acidity developed. The acid liberated is quantitatively equivalent to the amino-nitrogen combined.

Differential Sørensen titrations were performed in the following way. A small quantity of formaldehyde (approximately the theoretical quantity required to combine with all the amino-groups present) was added to the amino-compound and the acidity developed was titrated; increasing quantities of formaldehyde were then added and the acid liberated was determined after each addition.

In the following tables are given typical results obtained with a simple amino-acid (glycine), peptone, peptone infusion broth, a diphtheria toxin filtrate and a detoxified (formalinised) toxoid: 10 cc. of the neutralised solution were used in each case.

Table V. *Differential Sørensen titrations.*

40 % HCHO added (cc.)	0·1 *N* NaOH required to neutralise (cc.)		
	0·5 % glycine	2 % peptone	2 % peptone infusion broth
0·05	1·0	0·70	1·1
0·10	2·0	1·13	2·1
0·20	3·4	1·45	3·2
0·40	4·3	1·75	3·8
0·80	5·1	2·00	4·2
1·60	5·8	2·25	4·6
3·20	6·4	2·55	5·1

The equilibrium constant $K = \frac{[\text{glycine}]\,[\text{HCHO}]}{[\text{methylimino-compound}]}$ may be calculated from the above data. The mean value is 0·28 and divergences from this value are relatively small considering the nature of the experiments (limits 0·20 to 0·32).

Table VI.

Diphtheria toxin T. 800; Lf. 0·053 cc.		Toxoid F.T. 800; Lf. 0·056 cc.	
40 % HCHO added (cc.)	0·1 *N* NaOH to neutralise (cc.)	40 % HCHO added (cc.)	0·1 *N* NaOH to neutralise (cc.)
0·05	1·0	(0·04)*	(0)*
0·10	2·5	0·10	0·8
0·20	3·9	0·20	2·2
0·40	4·7	0·40	3·3
0·80	5·3	0·80	4·2
1·60	5·9	1·60	4·8
3·20	6·6	3·20	5·3

* Already present.

When the quantity of formaldehyde added is the amount theoretically sufficient to combine with all the amino-nitrogen present (usually about 0·05 cc. of 40 % HCHO added to 10 cc.) it is found that only some 15 % of the amino-nitrogen is combined, leaving about 85 % free. A very large excess of formaldehyde (over 6000 %) must be added before an approach to quantitative combination occurs. In the toxoiding process the amount of formaldehyde added (0·4 to 0·5 % of 40 % HCHO) is approximately the theoretical quantity to combine with all the amino-nitrogen. It is found, however, that even in the completely detoxified toxoid which has been incubated with formaldehyde for 6 weeks 85 % of the amino-nitrogen remains free and uncombined.

In Fig. 1 are plotted the results obtained with toxin T. 800 and the corresponding toxoid F.T. 800. It will be seen that the total titratable free amino-nitrogen of the toxoid is approximately 85 % of that of the toxin.

Furthermore, the same result has been obtained with every toxin and toxoid examined, and, not only so, but also with simple amino-compounds such as glycine, with peptone and with the medium in which the organisms are grown. It seems likely therefore that the same would be found true of the pure diphtheria toxin itself if it could be isolated. The same result was obtained when the Van Slyke method of determining amino-nitrogen was used in place

of the Sørensen titration method. It seems justifiable to assume therefore that only about one-sixth or one-seventh of the amino-groups in toxin disappear during the toxoiding process and that toxoiding may proceed by some altogether different mechanism.

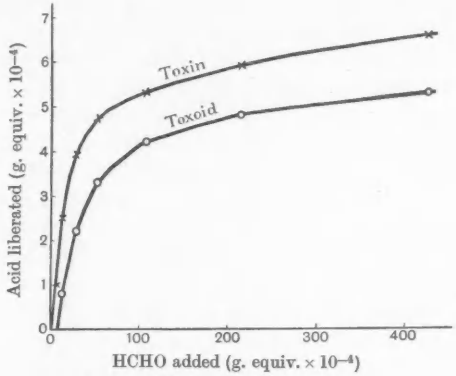

Fig. 1. Differential Sørensen titrations

This result is completely at variance with the conclusions of Kissin and Bronstein [1928, 1930] who claim that exactly 50 % of the free amino-groups in the toxin molecule disappear during the toxoiding process. Their experiments receive attention in the next section.

Acid titration curves.

Kissin and Bronstein's [1928, 1930] experiments were conducted as follows. Samples of toxin (5 cc.) were neutralised to litmus (Point I), *N* HCl was then added until the maximum precipitation was observed (Point II), then a further quantity of HCl was added until the reaction was acid to tropaeolin (Point III). If x cc. of acid were required to pass from Point I to II and y cc. to pass from II to III, the ratio x/y is known as the "acid quotient." The authors claim that the "acid quotient" of broth or of fresh diphtheria toxin is 2, whilst that of detoxified toxoid approximates to 1.

If attention is confined for the moment to the technical aspect of these experiments, a number of criticisms may be made. The use of litmus as a neutral point indicator is inadvisable owing to the wide range of colour change and the impossibility of accurate, reproducible readings. The point of maximum precipitation (II) is again not particularly well defined, there is a considerable range over which differences in precipitation are difficult to detect, and finally point III, the change of colour of tropaeolin (probably tropaeolin 00), is again susceptible to the inaccuracies involved in the observation of colour change. There is therefore considerable scope for errors in this titration method, particularly since the final "acid quotient" is the ratio of two amounts obtained by difference. A discrepancy of less than 0·1 cc. in the titrations is

sufficient to render the whole experiment useless. It must be pointed out that their experiments have not been confirmed by any other method.

Before going further it was decided therefore to repeat Kissin and Bronstein's experiments. It was impossible to obtain reproducible results of any value using litmus and tropaeolin 00 as indicators so electrometric titrations with the quinhydrone electrode were employed. Varying amounts of *N* HCl were added to 5 cc. samples of the toxin under examination and the p_H was determined electrometrically. A typical pair of experimental results is illustrated in the titration curves in Fig. 2. The neutral point to litmus was assumed to be p_H 6·2 (as stated by Kissin and Bronstein), that of tropaeolin 00 to be p_H 2·0, and the points of maximum precipitation of toxins and toxoids appeared to be not widely different and at about p_H 3·7. From the curves,

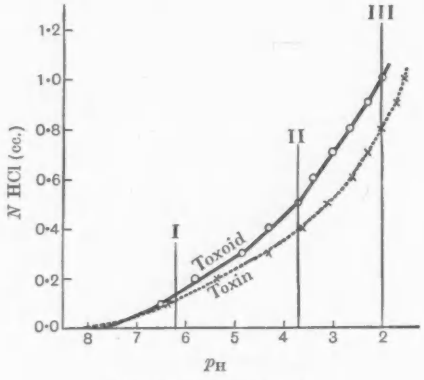

Fig. 2. Titration curves.

therefore, Kissin and Bronstein's "acid quotient" may be read off with a fair degree of accuracy. No appreciable difference between toxins and toxoids was found, in every case the "acid quotient" was between 1·3 and 1·5. Thus, when Kissin and Bronstein's experiments were performed with our toxins and toxoids under more accurate conditions their results were not confirmed.

The only other experiment reported by Kissin and Bronstein to support their view was the determination of the amino-nitrogen of toxin by the Van Slyke method before and after the toxoiding process. A 50 % diminution in free amino-nitrogen was observed. This is completely at variance with results here reported. As mentioned in the previous section a diminution of only 15 % in free amino-nitrogen was found, both by the Van Slyke and the Sørensen methods. Furthermore, this diminution occurred directly the formaldehyde was added to the toxin and was not a gradual process like that of toxoiding. It should be remarked that 3–5 minutes' shaking at 18–20° is generally considered sufficient for the completion of the reaction of nitrous acid with amino-groups. Kissin and Bronstein allow the reaction to continue for 30 minutes—

a period likely to result in hydrolysis of peptide linkings and hence to vitiate the measurement.

The theoretical interpretations placed by Kissin and Bronstein on the results of their experiments are also susceptible to critical examination. From the determination of acid quotients they conclude that the diphtheria toxin molecule contains two free amino-groups and one carboxylic acid group and that the toxoid molecule is identical but with only one free amino-group. The tacit assumption is made that in a toxic diphtheria culture filtrate there is present nothing but pure toxin, or, at any rate, that there are present no other substances likely to interfere with the titration. Of the substances in the toxic filtrate at least 99·5 % are not toxin at all, and the buffering power of peptone, phosphates, carbonates, etc. in various ranges of p_H is considerable. It is unwarrantable, therefore, to assume that the characteristics of the titration curve are attributable to the pure toxin and that conclusions regarding the constitution of the toxin molecule may be deduced therefrom. Kissin and Bronstein state that the original broth gives the same acid quotient as diphtheria toxin filtrates. This again should make for caution in drawing conclusions from the behaviour of the very complex mixture comprised in the filtrate after growth of the culture. If the toxic filtrate cannot be distinguished from the original broth by this method, then it seems unwise to attempt any deductions from the results regarding the constitution of the toxin.

An experiment with toxin purified by acid precipitation showed a diminution of amino-nitrogen from 0·04 mg. per 1 Lf. to about 0·00016 mg., showing that not more than $\frac{1}{250}$th of the amino-nitrogen in the toxic culture filtrate was attributable to the toxin itself.

Reducing effects of formaldehyde.

The oxidation-reduction condition of a reversible oxidation-reduction system may be observed by immersing an unattackable electrode in the solution. The potential difference set up at the electrode is dependent on the proportion of oxidised and reduced forms present of the substance studied. The more highly oxidising the system, the more highly positive is the potential and the more reducing the system, the more negative is the potential.

Formaldehyde itself does not produce an oxidation-reduction potential at an electrode. By itself it is inert electrochemically but when added, for example, to fresh milk an enzyme present has an activating effect, and the mixture develops reducing properties when incubated. These reducing properties may be observed by the reduction of methylene blue (Schardinger reaction) or by measurement of the reducing potential developed [Clark, Cohen and Gibbs, 1925].

When formaldehyde was added to diphtheria toxin and the mixture was incubated a slight reducing potential was developed. For example, in one case, 0·4 % of 40 % formaldehyde was added to a sample of toxin and the mixture was incubated. The original toxin had an oxidation-reduction potential of $E_h = 0{\cdot}34$ v. but the formalinised mixture developed slowly a

potential of $E_h = 0{\cdot}22$ v., at which level the potential was maintained for 10 days, the duration of the experiment.

It seemed possible therefore that the toxoiding process might be due to the maintenance of slight reducing conditions when the toxin was incubated with formaldehyde.

An alternative method of reduction was therefore attempted. It has been shown [Hewitt, 1930] that intense reducing conditions are established in diphtheria cultures during growth and that these are maintained after the cessation of active proliferation. After 9 days' incubation, therefore, a culture of *C. diphtheriae* (Park-Williams 8 strain) was sealed with melted vaselin, in order to prevent free access to air and to assist in the maintenance of reducing conditions. The incubation of the sealed culture was continued for 27 days. The filtrate of this culture was still highly toxic to guinea-pigs and the toxin-toxoid content, as measured by the Ramon flocculation method, had increased slightly during the incubation from 18 to 22 antigenic units per 1 cc. The flocculation time had also decreased slightly and the organisms proliferated actively when sub-cultured.

This method of reduction had therefore failed to produce the toxoiding process. That the toxoiding process is not due to the reducing action of formaldehyde is supported by the fact that although formaldehyde disappears during the toxoiding process the acidity corresponding to formic acid formation is not developed, as would be expected if the following formula expressed the process accurately:

$$\underset{}{H.CHO} + \underset{\text{toxin}}{T} + H_2O \longrightarrow H.COOH + \underset{\text{toxoid}}{TH_2}$$

If the formaldehyde, in its reducing effects, were oxidised still further to carbon dioxide,

$$HCHO + O_2 \longrightarrow CO_2 + H_2O,$$

there should be an increase of some 0·2 % in the CO_2 content of the toxin. Actually a decrease in CO_2 content from 0·13 % to 0·12 % was observed during the incubation with formaldehyde. During the toxoiding process a considerable proportion of the formaldehyde disappears but its transformation product has not been found.

Effect of other reagents.

Sodium hydrosulphite. To 20 cc. of toxin (T. 824, Lf. 0·04 cc.) were added 0·05 g. sodium hydrosulphite and *N* NaOH to neutrality. The mixture was incubated at 40° for 2 hours and allowed to stand in the incubator for one night. The mixture was rendered sterile by filtration through a Seitz filter and 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig. The animal died in 24 hours and *post mortem* examination showed typical diphtheritic toxaemic changes. This exposure to reducing conditions had not therefore led to disappearance of toxicity.

Hydrogen peroxide. 20 cc. of toxin (T. 824) were incubated with 0·04 cc. of hydrogen peroxide (20 vol. A.R.) at 40° for 2 hours. After standing overnight on ice 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig. The animal was dead in 16 hours and *post mortem* examination revealed typical acute diphtheritic toxaemia. The oxidation, therefore, had not caused disappearance of toxic effects.

Methyl alcohol. 20 cc. of toxin (T. 824) were incubated at 40° for 24 hours with 0·04 cc. of methyl alcohol (A.R.). 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig which was dead in 16 hours with typical acute diphtheritic toxaemia (*post mortem* indications). Methyl alcohol, which may be produced from formaldehyde by reduction, had therefore failed to remove the toxic properties of the toxin.

Conclusions.

In the conversion of toxin into detoxified toxoid the amount of formaldehyde added is approximately the quantity theoretically sufficient to combine with all the amino-nitrogen present. Under these conditions, however, only some 15 % of the total amino-nitrogen is combined, and even after 6 weeks' incubation some 85 % of it remains uncombined. A very large excess of formaldehyde is necessary before disappearance of free amino-nitrogen approaches completion. This is found to be the case not only with toxins and with broth but with peptone and the amino-acid, glycine. It seems probable therefore that the same would be found true of pure diphtheria toxin itself if it could be isolated. Since some six-sevenths of the amino-groups in the toxin molecule probably remain uncombined when this is converted into toxoid an alternative explanation of the effect of formaldehyde seems possible.

Kissin and Bronstein [1928, 1930] maintain that 50 % of the free amino-groups in the toxin molecule disappear during the toxoiding process and conclude that the action of formaldehyde is to remove those amino-groups (50 %) which are responsible for toxic effects but not for antigenic function or combining power. It was not found possible to confirm their results, and their experimental methods and theoretical conclusions are criticised.

As an alternative to the removal of free amino-groups, another function of formaldehyde was investigated. Formaldehyde is capable of developing reducing tendencies in the presence of a suitable activator. Oxidation-reduction potential measurements indicated some activation of formaldehyde when incubated with toxin, but another method of maintaining reducing conditions, namely, that of sealing a living diphtheria culture, failed to detoxify the toxin. Formaldehyde disappears during the toxoiding process but is not converted to formic acid, or CO_2, which is again evidence that it does not function as a reducing agent in the process.

Under the conditions employed reducing and oxidising agents and methyl alcohol failed to produce a detoxified toxoid.

Possible explanations of the toxoiding effect of formaldehyde, therefore, remain as follows.

1. The disappearance of a small fraction of the free amino-groups in the toxin molecule may result in detoxification without destruction of combining power with antitoxin, or antigenic activity.

2. The formaldehyde may cause union of two or more toxin molecules with formation of a compound of high molecular weight:

$$\underset{\text{toxin}}{TNH_2} + HCHO + \underset{\text{toxin}}{TNH_2} \longrightarrow \underset{\text{toxoid}}{THN - CH_2 - NHT} + H_2O$$

The compound, having the same general configuration as the original toxin, might possibly retain antitoxin-combining power without being able to exert toxic effects.

3. The formaldehyde may react with groups other than NH_2 in the toxin molecule.

4. The formaldehyde may itself undergo transformation, such as reduction, oxidation, polymerisation or combination with other broth constituents before reacting with the toxin. In this connection two facts are of interest, firstly the slow and gradual nature of toxoid formation and secondly that hexamethylenetetramine, acetaldehyde and trioxymethylene sulphite have been found to produce toxoid formation, whilst paraldehyde does not [Berthelot and Ramon, 1925; Hollande and Penn, 1928].

Although these hypotheses are entirely speculative they may provide a working basis for further investigation.

Summary.

1. Of the amino-nitrogen present in fresh diphtheria toxins some 85 % remains free in completely detoxified toxoids prepared by incubation with formaldehyde.

2. Maintenance of toxins under reducing or oxidising conditions failed to produce toxoid.

3. No evidence that toxoid formation is due to disappearance of free amino-groups, to reduction or to oxidation has been obtained. Possible mechanisms are discussed.

The author is deeply indebted to Dr R. G. White for his encouraging interest and to the laboratory staff for their cooperation.

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CXIII. THE SPECIFIC REFRACTION INCREMENTS OF SERUM-ALBUMIN AND SERUM-GLOBULIN.

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(1) Introduction.

The refractive index of a protein solution is greater than that of pure water, and, according to the observations of Reiss [1904] and Robertson [1912], the difference between the refractions of the protein solution and the solvent is equal to the protein concentration in g. per 100 cc. of solution, multiplied by a constant. This constant is termed the specific refraction increment or the specific refraction of the protein.

A critical account of the work of previous investigators on the refractions of the proteins in normal and in pathological sera has been published by Schretter [1926] who has drawn attention to the question as to whether the specific refraction increments show physiological or pathological variations. The results obtained by different observers show a wide range of variation in normal as well as in pathological sera, but further investigation is required in view of the risks of error due to the relatively low stability of the proteins and their association with lipins and other impurities.

The first problem investigated in this work is the preparation of a purified solution of crystalline horse serum-albumin under conditions which minimise the risk of error due to alterations in the protein. The purity and stability of the preparations have been tested by comparing their specific refraction increments after successive recrystallisations and after different periods of dialysis in accordance with the procedure adopted by Hopkins [1900] in establishing the individuality of egg-albumin by measurements of the optical rotation of the protein after successive recrystallisations.

The second problem is the application of Donnan's theory of membrane equilibrium to measurements of the refractive indices of serum-albumin and serum-globulin solutions which have been enclosed in collodion membranes and dialysed against phosphate mixtures of well-defined hydrogen ion concentration. An experimental investigation of the effects of the unequal distribution of salts on refraction is of value in determinations of the specific refraction increment of globulin, which cannot be dissolved in the absence of electrolytes.

The theoretical interpretation of the specific refraction increment of a protein is discussed in Section 11 in this work, and compared with determinations on amino-acids and calculations based on the refractivities of the constituent atoms.

(2) *A rapid method for the recrystallisation of horse serum-albumin.*

According to Svedberg and Sjögren [1928], serum-albumin once crystallised is a homogeneous substance of molecular weight 68,000, whereas recrystallised serum-albumin is a mixture of particles of different sizes, due to the decomposition of the protein. Their suggestion that the earlier determinations of 62,000 [Adair 1926], and 45,000 [Sørensen 1925, 1] refer to material partially decomposed will be discussed in a later communication.

In this work, an attempt has been made to determine the conditions for recrystallisation in which the protein remains unaltered. It is quite certain that the decomposition products referred to by Svedberg are not formed under all conditions, because preparations have been made in which the rotation of egg-albumin and of serum-albumin remains constant after a number of successive recrystallisations [Hopkins, 1900; Young, 1922]. This constancy of the rotation is of great importance as a proof of the stability as well as the purity of the preparations.

The methods used in this work have been tested by comparing the specific refractions of material crystallised from one to four times, instead of the specific rotations. Refractometric measurements are not quite so sensitive as the measurements of rotation, but they can be made on smaller volumes of material.

Recrystallised horse serum-albumin was prepared by a slightly modified form of the method of Hopkins. The preparations made under the conditions outlined below gave constant values for the specific refraction.

Normal sterile horse serum was mixed with an equal volume of saturated ammonium sulphate solution and stored for 1 day at 0°. The globulin precipitate was removed by centrifuging or filtration. The first crystallisation was brought about by the addition of acetic acid as described in detail by Young [1922], who recommends a preliminary washing of the serum with ether, a procedure which was followed in some of our preparations.

The solution was left to crystallise over-night at room temperature. If it is placed at 0°, amorphous matter may be deposited.

The following day the crystals were separated from the mother liquor by centrifuging, and then mixed with an approximately equal volume of a fluid prepared by adding 60 cc. of *M* sodium acetate solution and 40 cc. of *M* acetic acid to 100 cc. of a saturated solution of ammonium sulphate and again centrifuged. It is possible to wash the crystals with this fluid two or three times without much loss.

The quantities of reagents used in the process of recrystallisation given below refer to a preparation of about 2 g. of washed crystals, yielded by 100 cc.

of serum. The volume of water used to dissolve these crystals is about 50 cc. After filtration of the solution of albumin in order to remove the insoluble residue, which may consist of lipins [Young, 1922], 8 cc. of *M* sodium or ammonium acetate were added, followed by a volume of saturated ammonium sulphate solution equal to the sum of the volumes of the water and the sodium acetate (about 58 cc.). Finally, the acidity was adjusted by the addition of 10·6 cc. of a mixture composed of equal volumes of *M* acetic acid and saturated ammonium sulphate (1·33 cc. of this mixture per cc. of sodium acetate). The only reagent which need be added slowly is the mixture of acetic acid and ammonium sulphate, which may be mixed with the albumin solution in a period of about 15 minutes. Crystallisation takes place within 2 hours, and it may occur in a few minutes if the amounts of water and of ammonium sulphate are reduced, but under these conditions the crystals are very small. The solution must be stirred during the addition of acid and it is advisable to stir the solution during crystallisation.

The yield can be increased by leaving the preparation over-night at room temperature. The crystals are separated from the mother-liquor by centrifuging and redissolved and recrystallised by the same method. The yield after four crystallisations is about 1 g. per 100 cc. of serum if the process is completed in a period of 3 or 4 days, a period only slightly longer than that allotted for a single crystallisation in the experiment described by Svedberg.

The process of recrystallisation adopted in this work necessitates the preparation of more reagents than does Hopkins's original method, but it shortens the time required, because the volume of ammonium sulphate required can be calculated and added rapidly. In the original method, the ammonium sulphate must be added with caution, for a slight error of judgment in estimating the amount required may lead to the formation of amorphous precipitates.

(3) *The purification of albumin by dialysis against standard buffer solutions.*

The solutions of recrystallised albumin obtained by the method described above contained ammonium sulphate and other crystalloids which were removed by dialysis in collodion membranes. The method of dialysis adopted in this work differed from that described by previous workers, in that the solutions were subjected to a preliminary dialysis with a buffer mixture composed of sodium and potassium phosphates, before they were dialysed against distilled water, in order to convert ammonium albuminates into sodium or potassium albuminates. After the ammonia has been replaced by non-volatile bases, it is legitimate to use determinations of the ash content of the solutions as a test for the degree of purification effected by dialysis. The ash contents of solutions dialysed in different membranes for different periods are recorded in Table II, and it will be seen that the amounts of ash, which vary from 0·01 to 0·06 %, are too small to cause significant errors in refractometric measurements. For comparison with the experiments on aqueous solutions of albumin, a number of preparations at the physiological hydrogen ion concentration were made by

dialysing the protein against a phosphate mixture containing 0·8/15 g.-mol. of Na_2HPO_4 and 0·2/15 g.-mol. of KH_2PO_4 per litre of solution, at 0°.

(4) *Methods for the determination of the specific refraction increment of a protein.*

The specific refraction increment, α, is calculated from measurements of refraction and of the protein concentration as defined in formula (1):

$$[R' - R_s]/C = \alpha \qquad \text{.....(1).}$$

R' = the refractive index of the protein solution.

R_s = the refractive index of the solvent, at the same temperature.

C = g. of dry protein per 100 cc. of solution.

This method of defining the concentration is of significance, for if the concentration is expressed in g. per 100 g. of water or per 100 g. of solution, the specific refraction increment is not a constant, but a variable which is a function of the protein concentration.

The determinations of refraction were made by means of a Zeiss dipping-refractometer, which was immersed in the protein solution after the temperature of the fluid had become constant within 0·1°. The refraction of water was measured first, in all experiments, followed by determinations of the refraction of the outer fluid R'' and of the protein solution R'. In some experiments, the temperature of these solutions differed by 0·1° to 0·3° from the temperature of the water, and a correction was made of 0·02 refractometer scale division, or 0·0000078 in the refractive index, per 0·1°. The neglect of this correction would cause an error of 0·43 % in estimating the refraction difference $R' - R''$ for a 1 % protein solution.

In our observations on aqueous solutions of serum-albumin, it was found advisable to make readings of the refractometer for a period of at least 15 minutes. Solutions of albumin or of globulin containing salts usually gave accurate readings 1 or 2 minutes after the immersion of the refractometer in the beaker of solution. In the aqueous solutions, the refractometer readings in the first few minutes, before the boundary grows distinct, may be about 0·2 unit below the final steady values.

It should be observed that the temperature of the protein solution and of the control solution should agree within 0·1°; the absolute value of the temperature is not of great importance, for Robertson has found that the value of α is independent of temperature, over a range of 20°. Our determinations of the value of α, at temperatures between 15° and 22°, agreed within the limits of experimental error.

The following methods for the estimation of the protein concentration have been utilised in this work. For the purified solutions of serum-albumin, a known weight of the solution, contained in a platinum crucible, was dried to constant weight at 110° in a Lothar-Meyer air-oven. The weight of protein was calculated by deducting the weight of the ash. The concentration of the protein

was calculated from these measurements and from measurements of the density of the protein solution.

For mixtures of albumin and phosphates this method is inaccurate, because the orthophosphates may be converted into pyrophosphates in the process of ashing. In such cases the protein concentration was calculated from Kjeldahl nitrogen determinations. The calculation depends upon an accurate determination of the percentage of nitrogen in the protein, and in view of the discrepancies of about 2 % recorded by different workers, the nitrogen content has been redetermined on specially purified material as stated in Section 5.

(5) *The nitrogen content of serum-albumin.*

The elementary analyses of serum-albumin recorded by different observers show a range of variation in the percentage of nitrogen which is considerably greater than the probable error of Kjeldahl determinations. Values which have been obtained are recorded in Table I.

Table I. *Percentage of nitrogen in serum-albumin.*

Author	% nitrogen	Method
Dumas and Cahours*	15·7–16·5	—
Brittener*	15·6	—
Stärke [1881]	16·04	Washed with alcohol and ether
Michel [1896]	15·8–16·0	Washed with alcohol and ether
Michel [1896]	15·6–15·8	Dialysed against distilled water
Maximowitsch [1901]	15·9	Dialysed against distilled water
Goldschmidt and Kahn [1929]	15·9	Washed with alcohol and ether
Piettre and Vila [1921]	15·1–15·2	Washed with acetone and ether

* Cited by Michel.

In this work, the percentage of nitrogen in horse serum-albumin was determined by the following method. Solutions of crystalline albumin were dialysed under pressure against distilled water at 0° for periods of 9 to 21 days, glass-distilled water being used in the final stages. The density of the protein solution was then measured, and a known weight of the solution was dried to constant weight in a platinum crucible in a Lothar-Meyer air-oven at 110°. After the final weighing, the protein was ashed and the weight of ash was obtained. The nitrogen content of the solution was estimated by Kjeldahl determinations on aliquot portions. The results obtained are recorded in Table II.

Table II. *Percentage of nitrogen in horse serum-albumin, purified by crystallisation and dialysis.*

Preparation no.	No. of crystallisations	No. of days dialysis against water at 0°	Protein g. per 100 cc. solution	Ash g. per 100 cc. solution	Nitrogen g. per 100 cc. protein
3·1	1	9	8·47	0·0592	15·54
3·2 *a*	1	18	7·06	0·0372	15·67
3·2 *b*	1	18	7·05	0·0391	15·51
3·3	1	18	3·11	0·0142	15·80
4·0	4	10	2·90	0·0116	15·54
4·5	4	21	3·57	0·0097	15·62
				Mean	15·60

Our results agree therefore with those obtained by Michel [1896] and by Maximowitsch [1901] on protein purified by dialysis.

As stated by Michel, further investigation is required to elucidate the cause of the discrepancy between the ratios of carbon and nitrogen in the materials purified by washing with alcohol and by dialysis. For the purpose of refractometric investigations, it seems desirable to adopt the figure obtained on dialysed protein, because the specific refraction increments calculated on the assumption that the protein concentration equals the nitrogen concentration multiplied by $\frac{100}{15{\cdot}60}$ are then comparable with determinations on aqueous solutions of albumin, in which the protein content is determined by drying the solution to constant weight.

(6) *The refractions of aqueous solutions of crystalline horse serum-albumin.*

Measurements of the density, the refraction and the concentrations of solutions of horse serum-albumin purified by different methods are summarised in Table III. The ash content of the preparations ranged from 0·06 to 0·01 %. In the preparation with the highest ash content, the correction for the refraction of the ash is less than 0·7 % of the refraction of the protein. It is not practicable to make an exact allowance for the refraction of the ash, and the true values of the corrections may be appreciably smaller than the figures given in Table III, which represent the maximum values rather than the most probable values. The ash must have contained traces of sodium and potassium phosphates, since these salts were used in order to displace ammonium compounds by substances which could be estimated at least approximately by ash analysis. The maximum correction is obtained by assuming that the ash is entirely due to the potassium and sodium phosphates, in which case the refraction of the ash equals 0·00178 times its concentration. If it is assumed that the ash is carbonate, produced by the incineration of sodium or potassium salts of the protein, the correction is reduced.

In comparing the refractions at protein concentrations from 3 to 8 % and at temperatures from 14·7° to 20·1°, it will be seen that the results confirm Robertson's conclusion that the specific refraction increment α is independent of the protein concentration.

The most important point illustrated in Table III is the agreement of the values of α for preparations subjected to different degrees of purification. Young states that the rotation of once crystallised serum-albumin is different from that of material crystallised two or three times. The once crystallised material used in this work differed from Young's preparation in that it was washed with the acetate buffer mixture referred to in Section 1, and dialysed in collodion membranes of an improved type. A certain amount of precipitation took place during dialysis, which indicates that once crystallised serum-albumin is contaminated with impurities which are insoluble when the inorganic salts are removed by dialysis in highly permeable membranes.

Since the results for material crystallised once and four times are in close agreement, it appears that under favourable conditions the stability of serum-albumin is great enough to justify recrystallisation. The agreement of the results for material dialysed for 10 to 21 days affords additional evidence in favour of the stability of the preparations.

Table III. *Refractions of aqueous solutions of horse serum-albumin.*

Reference no.	HA 3·1	HA 3·2 a	HA 3·2 b	HA 3·3	HA 4·0	HA 4·5
No. of crystallisations	1	1*	1*	1	4	4
Dialysis time (days)	9	18	18	18	10	21
Density (g. per ml.)	1·0208	1·0173	1·0173	1·0064	1·0069	1·0087
Temp. of pyknometer	20·1°	18·2°	18·2°	20·1°	14·9°	14·7°
Dry protein (g. per 100 cc. solution)	8·4734	7·0594	7·0547	3·1053	2·902	3·5721
Ash (g. per 100 cc. solution)	0·0592	0·0372	0·0391	0·0142	0·0117	0·0097
Temp. of refractometer	18·7°	16·7°	16·95°	20·5°	14·7°	14·5°
Refractive index of protein solution, minus refractive index of water	0·015521	0·013011	0·012989	0·005664	0·005317	0·006633
Refraction due to ash	0·000105	0·000066	0·000070	0·000025	0·000021	0·000017
Refraction due to protein	0·015416	0·012945	0·012919	0·006539	0·005296	0·006616
a (average = 0·0018295)	0·001819	0·001834	0·001831	0·001816	0·001825	0·001852
Percentage deviation (average = 0·41 %)	0·575	0·246	0·082	0·0738	0·245	1·23

* Treated with ether.

The results of previous observers are summarised in Table IV for comparison with those obtained in this work.

Table IV. *Refractions of serum-albumin obtained by different workers.*

Author	Mean value	Range	Species	Fraction
Reiss [1904]	0·00201	—	Horse	Crystalline
	0·00183	—	Horse	Non-crystalline
Robertson [1912]	0·00177	—	Ox	Total
Rohrer [1922]	0·00177	0·00173–0·00180	Human and ox	Total
Schorer [1913]	0·00188	0·00175–0·00198	Human	Total
Starlinger and Hartl [1925]	0·00166	0·00137–0·00204	Human	Total
Schretter [1926]	0·00200	0·00191–0·00212	Human	Total

The range of variation in the determinations of the specific refraction increments summarised in Table IV is considerably larger than the probable error of the measurements of the refractive index, but there are other sources of error which deserve consideration. The preparation used by Reiss was dialysed for many days at room temperature, and it may have contained the protein derivatives of low molecular weight referred to by Svedberg. The other determinations in Table IV refer to uncrystallised albumin, from pathological as well as from normal sera. The methods adopted by Robertson, Rohrer and Starlinger and Hartl have been criticised by Schretter. Schorer and Schretter calculated the protein concentration from nitrogen determinations; it is

probable that their calculations are based on Stärke's determination that albumin contains 16 % of nitrogen. Guillaumin, Wahl and Laurencin [1929] adopt the figure 15·27 rather than 16. In this work the value 15·60 has been adopted. If Schretter's determination of α is recalculated on the assumption that the nitrogen content is 15·6 instead of 16 %, the result obtained is 0·00195. The experiments described below suggest that the difference between Schretter's value for total albumin and the value for the crystalline fraction obtained in this work is due partly to the existence of a non-crystalline fraction with a higher value for α and partly to impurities.

Sørensen [1925, 1] has stated that it is possible to separate crystalline serum-albumin into three fractions, characterised by differences in solubility. In one of our experiments, about half of the product was dissolved by washing with a diluted solution of ammonium sulphate, but this fractionation produced no appreciable change in specific refraction increment.

(7) *The refractions of solutions of sodium albuminate equilibrated with phosphate buffers.*

Serum-albumin in blood exists in the form of a sodium salt, and therefore it is desirable to supplement the measurements of the refraction of pure albumin with measurements of the refractions of albuminates. Preparations of albumin at the physiological hydrogen ion concentration were obtained by dialysis of the protein solutions against a standard phosphate buffer mixture, containing 0·8/15 g.-mol. of disodium phosphate plus 0·2/15 g.-mol. of potassium dihydrogen phosphate per litre of solution. The p_H' value of this solution is 7·38 at room temperature and 7·41 at 0°.

The protein solutions were enclosed in osmometers and dialysis was continued at 0° until the manometers gave constant pressures. The measurements of the refractive indices of the protein solution and of the dialysate were made at about 17·5°.

The relationship between the refractive index and the concentration of the protein can be determined by the calculation of the empirical coefficient α' defined by formula 2:

$$R' - R'' = \alpha' C \qquad \text{.....(2).}$$

R' = the refractive index of the protein solution.

R'' = the refractive index of the dialysate.

C = g. protein per 100 cc. solution, calculated from nitrogen determinations.

α' = an empirical coefficient, which may differ from the specific refraction increment α, on account of the unequal distribution of ions across the membrane. The mean values of α' and α are 0·00177 and 0·00183 respectively.

The results summarised in Table V and in Fig. 1 show that α' appears to have the same value for preparations from different sources and with different concentrations of the protein. Donnan's formulae, referred to below, show that α' may be increased by an increase in the protein concentration, but

Table V. *Refractions of different preparations of crystalline horse serum-albumin dialysed against a phosphate buffer* (p_H *7·4*). *In all these preparations the albumin was crystallised from solutions which had been extracted with purified ether to remove lipins.*

No.	C g. protein per 100 cc. solution	α' refraction increment	No. of crystal-lisations	Source
1	1·72	0·001757	1	Field labs. plasma
2	2·08	0·001760	1	,,
9	1·07	0·001798	1	Field labs. serum
8	2·47	0·001742	1	,,
7	2·49	0·001739	1	,,
6	3·54	0·001816	1	,,
5	4·86	0·001775	1	,,
19	1·48	0·001752	1	Burroughs Wellcome serum
18	1·50	0·001777	1	,, ,,
16	2·83	0·001811	1	,, ,,
17	2·99	0·001753	1	,, ,,
41	2·39	0·001755	4	Knacker serum
40	3·84	0·001770	4	,,
39	5·64	0·001759	4	,,
38	6·65	0·001769	4	,,
37	9·97	0·001791	4	,,

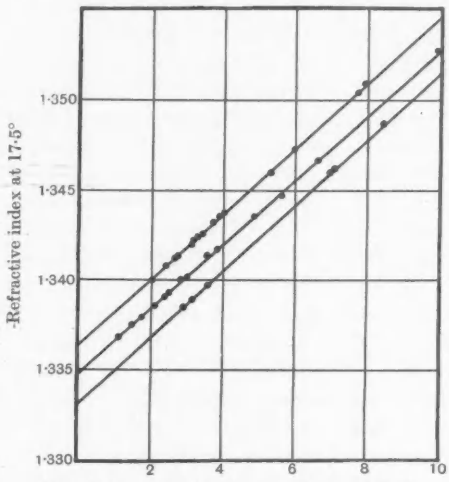

Fig. 1. Refractive indices of protein solutions at 17·5°.
Upper curve. Globulin, with 0·19 *M* KH_2PO_4 plus 0·009 *M* Na_2HPO_4.
Middle curve. Albumin, with 0·0133 *M* KH_2PO_4 plus 0·0533 *M* Na_2HPO_4.
Lower curve. Albumin dialysed against distilled water.

The lines in Fig. 1 were calculated by the following empirical formulae, in which the first constant equals the refractive index of the dialysate, and the second represents the value of α' or α.

Upper curve	$R = 1{\cdot}33639 + 0{\cdot}001815C$.
Middle curve	$R = 1{\cdot}33488 + 0{\cdot}00177C$.
Lower curve	$R = 1{\cdot}33320 + 0{\cdot}0018295C$.

within the range from 1 to 10 % of protein it appears that the variation is within the limits of the experimental error.

The degree of constancy in the values of α' shows that it is possible to calculate the concentration of the protein from measurements of the refractive index of the protein solution and of the dialysate by the application of formula (2). This calculation is independent of the theoretical interpretation of α'.

Schretter has pointed out that on account of the unequal distribution of ions across the membrane, it is not justifiable to assume that the refraction due to the protein can be calculated by subtracting the refractive index of the dialysate from the refractive index of the protein solution. He criticises the calculations of Rohrer [1922] which were based on the assumption that the concentration of salt is the same on both sides of the membrane. The error involved by the neglect of the salt distribution can be measured by comparing the values of α and α'. Under the conditions referred to in Table V, the difference between α and α' is about 3 %. The magnitude of the difference must depend upon the concentrations of hydrogen ions, of salts and of the protein.

The results of one experiment may be given, to show that under certain conditions, the difference α and α' must be relatively small. In this experiment, no. SA 43, a 10 % solution of sheep's serum-albumin was equilibrated with an ammonium phosphate buffer mixture at p_H 7·38 and 0°. The dialysate contained 0·115 g.-mol. of ammonia plus 0·0666 g.-mol. of phosphoric acid per litre. The protein solution contained 0·121 g.-mol. of ammonia plus 0·058 g.-mol. of phosphoric acid, plus 100 g. of protein per litre of solution.

The difference between the refractive indices of the dialysate and of water is equal to 0·00166 at 17·5°. The refraction of the ammonium phosphate inside the membrane must be approximately equal to 0·00166, multiplied by the distribution ratio, 0·058/0·0666, or 0·87. The total refraction due to inorganic ions must be greater than 0·0166 × 0·87, but it cannot exceed 0·00166 × 1·05, where 1·05 is the distribution ratio for ammonium ions. The difference between the refractive indices of the protein solution and of water is 0·02022, and by subtracting the maximum and minimum values for inorganic ions referred to above, it appears that the refraction due to the pure protein lies between 0·01848 and 0·01878. This conclusion was verified by a determination of α on an aqueous solution of sheep's serum-albumin, which gave the value 0·001856, or a refraction difference of 0·01856 for a 10 % solution. In this experiment, the value of α' is almost exactly equal to the value of α.

In the case of the experiments referred to in Table V, in which the distribution of ions was not determined by direct methods, approximate calculations of the concentrations of ions inside the membrane were made by a formula based on Donnan's theory.

$$S_{\text{inner}} = S_{\text{outer}}\,(1 - vx)\,.\,10^{-zE/54\cdot2}.$$

S_{inner} and S_{outer} denote the molar concentrations of a diffusible ion in the protein solution and in the dialysate, x, the number of g. protein per cc. of solution, v is equal to the effective volume of 1 g. of the protein (according to Weber and Nachmannson [1929] $v = 1$ cc)., n = the valency of the ion, and E is the membrane potential in millivolts at 0°. In the case of a 1 % solution of serum-albumin, the membrane potential is about 0·32 millivolt. The degree of accuracy of the formula has been investigated in a previous paper [Adair, 1928].

An approximate statement of the relationship between α' and α can be obtained by applying Donnan's equations for the distribution of ions across membranes, supplemented by the assumptions formulated in equation (3).

$$R' - R'' = (\alpha + \beta)\, C + \alpha_K\, (K_{inner} - K_{outer}) + \alpha_{Na}\, (Na_{inner} - Na_{outer}) \ldots (3).$$

C = g. pure protein per 100 cc. of solution.

β is a factor approximately equal to 0·00003, which represents the effect of the sodium or potassium combined with the protein at p_H 7·4 (approximately 0·00043 equivalents per g. protein). The factor β was determined by comparing the refractions of aqueous solutions of the pure protein and of sodium proteinates.

α_K is an empirical coefficient, = 0·016, defined by a formula resembling equation 1.

K_{inner} = the concentration of KH_2PO_4 in the protein solution in g.-mols. per litre of solution.

K_{outer} = the concentration of KH_2PO_4 in g.-mols. in the dialysate.

α_{Na} is an empirical coefficient = 0·0279.

Na_{inner} and Na_{outer} refer to the concentrations of Na_2HPO_4. In the dialysate the molar concentrations of KH_2PO_4 and Na_2HPO_4 are equal to 0·01333 and 0·05333 respectively. In the protein solution their concentrations are a function of the protein concentration. In the case of a 1 % solution ($C = 1{\cdot}0$), the concentrations of KH_2PO_4 and Na_2HPO_4 are approximately equal to 0·01303 and 0·05142 respectively. These figures are provisional estimates based on the application of Donnan's formulae.

The value of $R' - R''$ is equal to 0·0177 in the case of a 1 % solution, and by applying formula (3) to the experimental data given above, it is possible to calculate the value of α. The result obtained, 0·00180, is in fairly close agreement with the more accurate value 0·00183 obtained by direct measurement. This agreement is of significance in the determination of the specific refraction increment of globulin, in which it is necessary to rely upon indirect methods for the determination of the specific refraction increment.

(8) *The effects of purification processes on the refraction of sodium albuminates.*

In the previous section it has been shown that the refraction increment α', defined by formula (2), is a constant in the case of solutions of albumin which

have been purified by washing with ether. The measurements on material which has not been purified by ether, recorded in Table VI, show a wider range of variation. The mean value of a' for the once crystallised albumin is 0·00183. The mean for twice crystallised and four times crystallised albumin is 0·00180. Hopkins [1900] and Young [1922] have observed that crystals of serum-albumin are associated with lipins, and it is not improbable that the value 0·00183, which exceeds by 3·3 % the refraction of the protein purified by ether, is due to the association of the protein with traces of lipins. The observations on protein crystallised four times agree better among themselves, and the average value 0·00180 is closer to 0·00177, the result for material treated with ether. Theoretically, the use of ether may be open to objection on the ground that it may alter the properties of the protein, but the results recorded above indicate that its use is justifiable.

Table VI. *Refractions of different preparations of crystalline horse serum-albumin dialysed against phosphate buffer at* p_H *7·4. The preparations differ from those in Table V in that the solutions were not treated with ether.*

No.	C g. protein per 100 cc. solution	a' refraction increment	No. of crystallisations	Source
13	1·94	0·00184	1	Burroughs Wellcome serum
12	2·19	0·00184	1	,, ,,
15 *a*	3·66	0·001842	1	,, ,,
14	4·64	0·001775	1	,, ,,
21 *a*	1·96	0·00184	1	,, ,,
22 *a*	1·97	0·00179	1	,, ,,
20	3·62	0·001816	1	,, ,,
35	2·44	0·001821	1	,, ,,
34	4·61	0·001823	1	,, ,,
33	6·60	0·001872	1	,, ,,
36	7·41	0·001843	1	,, ,,
32	9·14	0·001845	1	,, ,,
23	2·05	0·00177	2	,, ,,
24	1·86	0·001795	4	,, ,,
25	1·96	0·001812	4	,, ,,
27	3·81	0·001804	4	,, ,,
26	3·87	0·001813	4	,, ,,
28	1·58	0·001812	4	,, ,,
29	1·60	0·001820	4	,, ,,
30	2·82	0·001784	4	,, ,,
31	3·13	0·001809	4	,, ,,

(9) *The refraction of uncrystallised albumins from horse serum and sheep serum.*

The mother-liquor from two preparations of albumin crystals was dialysed against the phosphate buffer mixture defined in section (3). The value of a' for the first preparation was 0·001821. In the second preparation, the mean value of a' was 0·00188, a figure which is 6·2 % greater than the refraction increment of the crystallised albumin. Since a part of the protein in the mother-liquor must be crystalline albumin remaining in solution, it may be inferred

that the mother-liquor contains a protein with a refraction increment greater than 0·00188.

One experiment was made on albumin from sheep's plasma. The globulin was removed by half-saturation with ammonium sulphate and acetic acid was added. The albumin formed an amorphous precipitate instead of the crystals which appear when horse serum is used. The precipitate was redissolved and reprecipitated and then dialysed against distilled water. The specific refraction increment α, defined by formula (1), was 0·00185. The protein concentration in this experiment was calculated from nitrogen determinations. Within the limits of experimental error, this agrees with the results for crystalline horse serum-albumin recorded in Table IV.

Sheep serum, like horse serum, may contain substances with a higher refraction than the protein crystallised or precipitated by acid, for the total albumin in one preparation gave a value of 0·00194 for α', the refraction against a phosphate mixture. This preparation was not treated with ether, and therefore an appreciable fraction of the refraction increment may be due to lipins.

(10) *The refractions of isoelectric globulins and of sodium globulinates equilibrated with phosphate buffers.*

Direct determinations of the specific refraction increment for globulin have been made by Robertson and other workers, who have precipitated globulin by dilution or dialysis and redissolved the precipitated globulin by the addition of electrolytes. Robertson worked with very dilute solutions, as the opalescence of his preparations made it impossible to determine the refractive indices in concentrated solutions.

Preliminary experiments made in this work indicated that clear solutions of globulin could be obtained from material precipitated by ammonium sulphate and purified by dialysis against standard phosphate mixtures. The coefficient α' can be determined directly by measuring the refractive indices of the protein solution and the dialysate, and the value of α can then be calculated by formula (3), which allows for the effects of the unequal distribution of ions.

Svedberg and Sjögren [1928] state that globulin is an unstable substance, rapidly decomposed during the processes of fractionation into "euglobulin" and "pseudoglobulin." In order to minimise the risk of error due to decomposition of the protein, a rapid method of preparation was adopted in this work. The precipitate of globulin obtained by the addition of an equal volume of saturated ammonium sulphate to normal horse serum was separated by centrifuging. It was purified by the addition of water and reprecipitation with ammonium sulphate not more than three or four times, then cooled to 0° and dialysed in collodion membranes against standard phosphate mixtures. The protein appears to be fairly stable at 0°, because the values of α' for different preparations dialysed for different periods are in close agreement.

Table VII. *Nitrogen content of horse serum-globulin.*

No.	C g. dry protein per 100 cc.	g. nitrogen per 100 g. dry protein	Difference. Refractive index protein solution minus dialysate	α' calculated from dry weight determinations
G 7	3·162	15·24, 15·19	0·00582	0·00184
G 16	12·069	15·05, 15·06, 15·00	0·02219	0·00184
G 17	7·96	15·07, 15·06, 15·06	0·01452	0·00183
G 18	5·94	15·22, 15·24	0·01086	0·00183
G 21	10·421	15·16, 15·18	0·01909	0·00183

In five experiments, given in Table VII, the protein concentration C in g. dry protein per 100 cc. of solution was determined by the method of Devoto, as described by Hopkins and by Young. The removal of the last traces of sulphate from the heat-coagulated protein may require 10 days' washing in distilled water. For most of the time the material was kept at 0°. The nitrogen content of these solutions was determined by Kjeldahl's method and the percentages of nitrogen in the dry protein are given in Table VII. The mean value obtained for total globulin is 15·13 g. nitrogen per 100 g. dry protein.

Table VII gives the values of α' calculated from the dry weight determinations and measurements of refractive indices of solutions of globulins equilibrated with a mixture containing 0·19 g.-mol. of KH_2PO_4 plus 0·009 g.-mol. of Na_2HPO_4 per litre of solution. The p_H of this solution, 5·35, is in the isoelectric region.

Table VIII. *The refractions of globulin from horse serum equilibrated with phosphate buffer* p_H *5·35.*

No.	C	α'	No.	C	α'
1	3·30	0·001813	11	7·74	0·001817
2	3·39	0·001803	16	12·00	0·001848
3	4·02	0·001828	17	7·93	0·001832
6	5·33	0·001796	18	5·98	0·001817
7	16·98	0·001827	19	3·92	0·001825
8	15·91	0·001826	20	4·01	0·001805
9	11·92	0·001762	21	10·45	0·001828
10	9·84	0·001797	22	2·43	0·001813

In the experiments summarised in Table VIII the values of α' have been calculated from nitrogen determinations.

The mean value of α' for the isoelectric protein is equal to 0·001815. According to Donnan's theory, the concentration of the diffusible ions should be the same on both sides of the membrane at the isoelectric point. It is, however, necessary to make a correction for the volume of the protein. In the case of an ideal solution of an isoelectric protein, the correction can be made by the following formula:

$$\frac{R' - R''}{C} = \alpha' = \alpha - 0{\cdot}01 v_1 (R'' - R_{H_2O}).$$

v_1 = the volume occupied by 1 g. protein, plus the volume of its water of hydration. According to Weber and Nachmannson [1929], the effective volume of 1 g. globulin is 1·3 cc.

$R'' = 1{\cdot}33639$ at 17·5°.

$R_{H_2O} = 1{\cdot}33320$ at 17·5°.

In a 1 % solution of the protein the concentration of the diffusible salts is diminished by 1·3 %. By applying the formula given above, it appears that $\alpha = \alpha' + 0{\cdot}000041 = 0{\cdot}001857$.

In addition to the measurements of the isoelectric point, observations have been made on globulin equilibrated with the phosphate mixture used in the work on albumin, as stated in Table IX.

The mean value of α' for this medium is 0·00181. If it is assumed that the difference between α and α' for globulin is the same as that observed for albumin (0·00006) it follows that $\alpha = 0{\cdot}00187$.

Table IX. *The specific refraction increments of globulin from horse serum and sheep serum, equilibrated with phosphate buffer mixture.* $\frac{0{\cdot}8}{15}$ *M* Na_2HPO_4 *plus* $\frac{0{\cdot}2}{15}$ *M* KH_2PO_4. p_H *7·4.*

No.	g. protein per 100 cc. solution	α'	Species	Notes
100	7·80	0·00178	Sheep	—
101	14·00	0·00180	Sheep	—
G 4	2·75	0·00182	Horse	Serum treated with ether
G 5	2·72	0·00181	Horse	Serum treated with ether
G 24	1·64	0·00181	Horse	Stored 8 months

Table X. *Determinations of the specific refraction increment of globulin.*

Author	Range	Mean	Mean re-calculated	Species
Reiss	—	0·0023	—	Horse
Robertson	—	0·00229	—	Ox
Rohrer	0·00172–0·00180	0·00177	—	Human and ox
Schorer	0·0023 –0·0027	0·00245	0·00232*	Human
Starlinger and Hartl	0·00214–0·00312	0·00265	—	Human
Schretter	0·00206–0·00234	0·00219	0·00208*	Human
Adair and Robinson	0·00180–0·00189	0·00186	—	Horse

* These recalculations have been made because it is probable that Schorer and Schretter used Hammarsten's estimate that globulin contains 16 % of nitrogen in calculating the protein concentrations from Kjeldahl determinations. The value found in this work is 15·13 %.

(11) *The theoretical significance of the specific refraction increment of a protein.*

An approximate statement can be made of the theoretical correlation of the specific refraction increment and the refractive index of the pure protein. The refractive index has been computed by Robertson [1918], whose formula is based on the assumption that Gladstone's formula [Gladstone and Dale, 1863] for the refractions of mixtures is applicable to protein solutions, and that the specific volume of a dilute protein solution is equal to unity.

Gladstone's formula is

$$(R - 1)\, v = (R_p - 1)\, v_p x + (R_s - 1)\, v_s (1 - x).$$

R, R_p and R_s denote the refractive indices of the solution, the pure protein and the solvent.

v, v_p and v_s denote the specific volumes of the solution, the protein, and the pure solvent.

x = g. of protein per g. of solution.

Robertson's assumption that $v = 1{\cdot}0$ leads to the conclusion that

$$\alpha = 0{\cdot}01\,(R_p v_p - v_p - R_s + 1).$$

A more accurate formula, numbered (4) below, can be obtained by assuming that the specific volume of the solution is equal to $xv_p + (1-x)\,v_s$. Gladstone's formula is then equivalent to the following simple expression:

$$R - R_s = 0{\cdot}01 C v_p\,(R_p - R_s) \qquad \text{.....(4).}$$

C = g. solute per 100 cc. of solution = $x \times 100/v$.

If it is assumed that the solute is combined with g g. of water per g. of dry material, the formula requires modification

$$R - R_s = 0{\cdot}01 c\,(1+g)\,v_p'\,(R_p' - R_s) \qquad \text{.....(5).}$$

R_p' = refractive index of hydrated solute of specific volume v_p'.

C = g. anhydrous solute per 100 cc. of solution.

According to the data of Sørensen [1925, 2], $g = 0{\cdot}303$ for serum-albumin and 0·343 for serum-globulin.

By applying formula (5) to the values of α for albumin and globulin, 0·00183 and 0·00186 respectively, it appears that the refractive indices of the pure protein hydrates are approximately 1·514 and 1·511. These figures are approximations, because it is not yet possible to make allowances for solution-contraction volumes and for alterations in the refractivity of water [Fajans, 1927]. In the case of inorganic salts, the refractive index calculated from observations on solutions may differ by more than 3 % from the refractive index of the crystalline solid.

It is possible that the water of hydration in the protein crystals differs according to the composition of the mother-liquor, and the refractive indices of the crystals should show corresponding variations.

(12) *The specific refractivity of amino-acids and proteins.*

Gladstone and Dale define the specific refractivity of a substance by the formula $(R_p - 1)\,v_p$. An alternative definition, based on the formula of Lorentz and Lorenz, has been used by Brühl [1891], but the simpler equation of Gladstone and Dale is sufficient for the purpose of this work. The materials required for the calculations of the specific refractions of certain amino-acids are given in Table XI. The values of α have been calculated for comparison with the values of α given for the proteins. It was observed that the amino-acids differ from the proteins in that α is not a constant, but tends to increase as the degree of dilution is increased. A similar increase takes place

Table XI. *The refractions of solutions of amino-acids.*

Acid	Acid weight (g.)	Solvent weight (g.)	Temp. ° C.	Density	Refraction difference	α
Glycine	0·7505	10·0086	15·3	1·02924	0·01286	0·00179
Glycine	0·7505	9·9899	17·8	1·02886	0·01282	0·00178
Glycine	0·7500	9·9806	15·5	1·0294	0·01285	0·00179
Alanine	0·8908	9·9983	16·4	1·02564	0·01429	0·00171
Alanine	0·8908	9·9807	14·9	1·0260	0·01431	0·00171
Valine	0·188	9·9827	21·1	1·00216	0·00326	0·00176
Valine	0·3904	9·9872	21·5	1·00626	0·00664	0·00175
Tryptophan	0·102	9·9723	17·8	1·00166	0·002547	0·00251
Tryptophan	0·072	9·9810	20·9	1·00004	0·001813	0·00253

in solutions of electrolytes such as sodium chloride, in which the partial specific volume of the solute diminishes as the solution is diluted.

It is known that under certain conditions, the specific refractivity of an organic compound can be calculated from the specific refractivities of its constituent atoms. The calculated values for the amino-acids and for anhydrous serum albumin are given in Table XII, col. 1, for comparison with the observed values, based on determinations of refraction and density of the solutions recorded in Table XI.

Table XII. *The specific refractivities of amino-acids and of serum-albumin.*

Acid	Calculated specific refractivity	Observed specific refractivity
Glycine	0·37	0·37
Alanine	0·40	0·40
Valine	0·43	0·43
Tryptophan	0·42	0·49
Albumin	0·42	0·43

The calculated values are based on the values for the elements given by Smiles [1910], namely, C = 0·383, H = 1·488, N = 0·343, O = 0·203, S = 0·422. The calculation for albumin is based on an analysis given by Michel: C, 53·04 %; H, 7·1 %; N, 15·71 %; O, 22·29 %; and S, 1·86 %; these figures refer to the anhydrous protein. The calculation of the specific refractivity of the anhydrous protein is based on a determination of its apparent specific volume in an aqueous solution, 0·739 cc., computed from the density determinations given in Table III.

The results for glycine, alanine and valine agree with the theoretical calculation. The figure for tryptophan is approximately 17 % in excess. This excess is characteristic of unsaturated linkages in general. In benzene, for example, the observed specific refractivity is nearly 25 % greater than that calculated from the refractivity of the atoms. In the case of serum-albumin the observed value is about 2·9 % greater than the calculated. It is not improbable that the proportion of phenylalanine and other acids containing the benzene ring is sufficient to account for the discrepancy.

SUMMARY.

1. Horse serum-albumin has been prepared and recrystallised by a rapid method based on the original method of Hopkins. The nitrogen content has been redetermined as 15·6 %, a figure agreeing with the earlier estimates of Maximowitsch and of Michel for dialysed albumin.

2. The purity and stability of the preparations have been tested by measurements of their refractive indices, and it has been found that the specific refraction increment is a constant, unaffected by recrystallisation. In aqueous solutions the specific refraction increment of crystalline serum-albumin is 0·00183.

3. The refractive indices of solutions of alkali albuminates, equilibrated with phosphate buffers, have been measured and represented by a simple empirical formula, which can be used for calculations of the protein concentration from refractometric measurements. A calculation of the specific refraction increment of the protein from measurements of the refractive indices of the solution and the dialysate has been made by the application of Donnan's theory of membrane equilibrium.

4. The specific refraction increments of the uncrystallised fractions of albumin are larger than 0·00183.

5. The preparation of clear solutions of horse serum-globulin is described. The nitrogen content of total globulin has been redetermined as 15·13 %. The refractive indices of solutions of isoelectric and of alkaline globulins equilibrated with phosphate buffers have been measured and the specific refraction increment of the protein calculated, after making corrections for the effects due to the unequal distribution of ions. The mean value obtained for total globulin is 0·00186.

6. Preliminary determinations indicate that the specific refraction increments of both albumin and globulin prepared from sheep's serum are approximately equal to 0·00185.

7. Measurements have been made of the refractive indices of solutions of four amino-acids, namely, glycine, alanine, valine and tryptophan.

8. The theoretical significance of the specific refraction increment of a protein is discussed.

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CXIV. THE ACTION OF PEPSIN ON GELATIN.

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THERE can be little doubt that the chief chemical change which accompanies the digestion of protein by the enzymes of the pancreas and intestine and by the endocellular proteases is the hydrolysis of acid amide (*i.e.* peptide) linkages. This conclusion rests securely upon the fact that carboxyl and amino-groups are liberated in large numbers and in equivalent amounts. The magnitude of these chemical effects, moreover, has been found to run more or less parallel with the extent of the "physical digestion" of the protein which is betrayed in changes in solubility, diffusibility, viscosity, etc. In these respects, however, the behaviour of pepsin is peculiar. Pepsin is able to accomplish quite extensive degradation of protein without effecting any significant increase in titratable groups. This situation has led, from time to time, to the suggestion that the specific character of peptic digestion lies in the disintegration of the protein molecule into smaller fragments which were united, in the original molecule, by forces other than those of primary valency. Such a view is difficult to prove or to deny. It may be suggested, however, that it is a view whose utility becomes questionable once it has been definitely established that protracted digestion of protein is accompanied by regular and orderly increases in acidic and basic groups.

That such increases do occur has been demonstrated repeatedly, but the evidence respecting their orderliness has been somewhat conflicting. In particular, the report has frequently been made that the carboxyl groups liberated are in considerable excess of the amino-groups simultaneously set free. This has led to the impression that there may occur in the protein molecule linkages in which —COOH is united to some other group than primary $-NH_2$.

Within recent years there has been a revival of interest in the question owing to general recognition of the fact that its solution has an important bearing upon the general problem of protein structure. In 1926 two papers [Steudel, Ellinghaus and Gottschalk, 1926] appeared in which were reported studies of the peptic digestion of fibrin, edestin, vitellin, caseinogen, serum-albumin, serum-globulin, gluten and gelatin. In every case the authors found that the ratio of liberated groups, $\frac{COOH}{NH_2}$, exceeded unity. In the early stages of digestion it sometimes attained values as great as 7. Waldschmidt-Leitz and Simons [1926] also observed an excessive liberation of —COOH in the

cases of gelatin, gliadin and zein. In the digestion of caseinogen, egg-albumin and ricinus-globulin, on the other hand, the hydrolysis ratio approximated to unity. Meanwhile Steudel and Ellinghaus [1927] confirmed their original observations in a further paper. Felix and his collaborators [Felix and Harteneck, 1927; Felix and Muller, 1927] reported that the peptic digestion of thymus histone was accompanied by an equivalent increase in acidic and basic groups. Discrepancies between the increases in total acid-binding power and in free $—NH_2$ groups led them, however, to suggest the participation of the guanidine group of arginine in linkages with —COOH. This suggestion received no support from Waldschmidt-Leitz and Kunstner [1927], who arrived at the conclusion that the only important chemical changes which accompany peptic digestion are the hydrolysis of peptide linkages. This view was further supported by the observations of Felix and Buchner [1927] on haemoglobin, of Weber and Gesenius [1927] on caseinogen and of Sørensen and Katschioni-Walther [1928] on caseinogen, gliadin and gelatin. These last authors discuss in detail a number of errors which attend the application to protein solutions of the usual methods of determining —COOH and $—NH_2$ groups. They conclude that it has been an inadequate appreciation of these errors which has been responsible for the various observations in which the numbers of equivalents of liberated —COOH and $—NH_2$ have been found to be unequal.

The balance of evidence clearly favours the view that pepsin, like other proteolytic enzymes, is a true peptidase. It is, however, disturbing when different observers, using similar methods of analysis, arrive at such conflicting results. There remains a feeling that the methods may not be well adapted to the purposes for which they have been employed.

The determination of —COOH has always been made by titration of the digests with alkali in the presence of alcohol, formaldehyde, acetone or mixtures of these solvents. For the determination of $—NH_2$ groups, use has been made of some form of titration with acid such as that of Linderstrøm-Lang [1928], of Folin's colorimetric method or of the nitrous acid method of Van Slyke. The uncertainty of the two latter methods when applied to proteins is well known. It must be conceded, also, that the various titrimetric methods are equally empirical since they depend upon the titration of acidic and basic groups to arbitrary end-points in mixed solvents in which the controlling acid-base equilibria have not been adequately defined. It is to be observed, however, that their use rests upon the assumptions that such equilibria do exist and that the reaction of proteins and their digestion products with acids and bases are stoichiometric under the empirically chosen conditions. In view, therefore, of the success which has attended the quantitative treatment of proteins as ampholytes, it is surprising that no attempt appears to have been made to establish the characteristics of peptic digestion by direct electrometric titration of the aqueous digests. In the present paper such an attempt will be described.

Experimental.

Gelatin was chosen as substrate since it offered an unusual combination of advantages. It is readily and extensively digested by pepsin. It is soluble over the whole experimental p_H range and its digestion is not complicated by the separation of insoluble plastein material. The general form of its titration curve is well known and the interpretation of changes which are found to occur upon digestion is not confused by the presence of such ionising groups as the phenolic hydroxyl and thiol groups or by large numbers of amide groups. It has, moreover, been in the case of gelatin that many of the conflicting results, to which reference has been made, have been obtained.

Isoelectric gelatin was prepared by the method of Loeb from three different commercial samples. In the various experiments, also, three different commercial samples of pepsin were employed. The composition of the initial digest mixtures was varied with respect to the concentration of gelatin (gelatin-nitrogen 0·30–0·84 %), the concentration of pepsin (pepsin-nitrogen 0·003–0·03 %) and the initial p_H (1·6–3·1). The ratio of pepsin-nitrogen to gelatin-nitrogen did not, in any experiment, exceed 0·09 and was reduced in some below 0·01. In the latter cases, the increases in acid-binding capacity which accompanied digestion were in considerable excess of the total nitrogen of the pepsin. Consequently there was no possibility that changes in the enzyme material were responsible for the effects observed. The digestion mixtures were preserved with toluene and incubated at 38°. At intervals up to 14 days samples were removed for analysis. All observations were controlled by determinations on similar samples from identical mixtures containing inactivated enzyme. The latter was prepared either by heating the solution of enzyme or by rendering it alkaline to p_H 9 for 15 minutes and then adding an equivalent of acid. The latter method had the advantage of avoiding the introduction of coagulated matter into the control mixtures. Some or all of the following determinations were made on each analytical sample: the hydrogen electrode titration curve, the formaldehyde titration (Sørensen), amino-nitrogen (Van Slyke), viscosity, time of gelation at 0° at the isoelectric point, and nitrogen soluble in four volumes of alcohol at the isoelectric point.

The last three determinations were intended to control the rate of change in the physical properties of the hydrolysate. In this way it was hoped to provide a commentary upon the more intimate chemical changes revealed by analysis.

Hydrogen electrode titration.

Platinised or palladinised platinum electrodes were employed. The reference electrode was a saturated calomel half-cell connected to the unknown solution by a saturated potassium chloride bridge. The calomel cell was standardised against 0·1 *M* hydrochloric acid, the p_H of the latter being taken to be 1·08 between 25 and 38°. Two methods of titration were followed.

Method I. To small aliquot volumes of the digest were added varying amounts of standard sodium hydroxide and the mixture was diluted to a standard volume. The hydrogen electrode potentials of these mixtures were determined in an electrode vessel of the type designed by Clark. New electrodes were brought into service at the first indication of sluggish reaction or lack of reproducibility.

Method II. The second method was more economical of time and material and appeared to be equally accurate. 20 cc. of digest were placed in a small titrating bottle containing a Bunge electrode. Such a volume of sodium hydroxide was added as would bring the mixture to about p_H 11 and the air space was displaced with purified hydrogen. The alkali served to destroy the enzyme and so insure that no further digestion occurred during the progress of a titration. The mixture was then titrated with 0·1 *M* hydrochloric acid, potentials being determined after each addition. A new electrode was substituted whenever there was reason to question the activity of that in use. In general, one electrode survived a complete titration. Initial equilibrium required about 30 minutes but adjustments to additions of acid were usually complete within a few minutes.

In the second method of titration it was anticipated that loss of fluid through frothing would occur. As a matter of fact frothing never became uncontrollable. Since, however, a modified electrode was devised to meet the contingency its description may be of interest. The modification consisted in providing a by-pass to the hydrogen supply to the electrode. This may conveniently take the form of the glass portion of another Bunge electrode immersed in water to a depth just less than that to which the hydrogen electrode is immersed in the experimental solution. When the gas supply is turned on the levels of the fluids in the two electrode tubes pulsate together but hydrogen escapes only from the by-pass. Excessive agitation of the protein solution is, thereby, avoided. It is important that the gas train be constructed of metal or other tubing not permeable to gases and that it be liberally swept out with hydrogen at the beginning of the experiment. A few bubbles of hydrogen, also, should be allowed to escape into the solution at short intervals, this being effected by momentarily closing the by-pass. This precaution is taken to meet the tendency of traces of heavier gases to be displaced downwards to the electrode surface. The modified electrode worked well in practice.

Titration curves.

The "corrected equivalents of base" (b') in combination with 1 g. of gelatin at the various determined p_H values have been calculated from the relation

$$b' = b - a + h - oh,$$

where a and b are the respective concentrations of added hydrochloric acid and sodium hydroxide and h and oh are the concentrations of hydrogen and

hydroxyl ions respectively. The last two were calculated from the observed hydrogen ion activities with the aid of the relations

$$p_h = p_H - \log \gamma \text{ and } p_H + p_{OH} = p_{k_w}.$$

The activity coefficient of the ion concerned, γ, was calculated from the simplified equation of Debye and Huckel [Simms, 1926]

$$\log \gamma = 0{\cdot}3\sqrt{\mu}.$$

In computing μ, the ionic strength of the solution, the contribution of the protein ions was arbitrarily ignored.

For economy of presentation a graphic method of analysis of the titration curves may be adopted. Only one experiment will be quoted in detail. Fig. 1

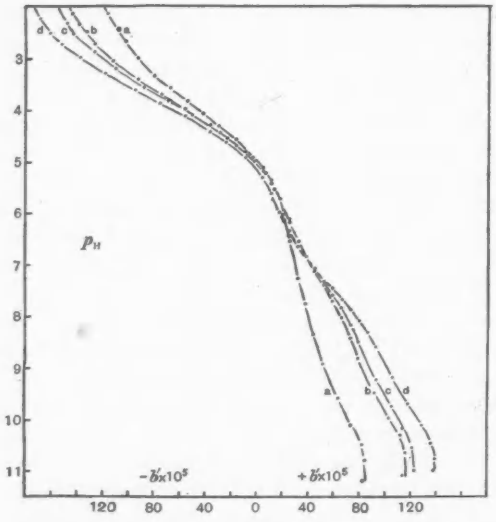

Fig. 1. Titration curves of peptic digests of gelatin. (*a*) Curve of 24 hours' control sample. (*b*) Curve of 24 hours' digest sample. (*c*) Curve of 48 hours' digest sample. (*d*) Curve of 7 days' digest sample.

presents the titration curves of a digestion mixture at various stages of digestion. The equivalents of acid ($-b'$) and of base ($+b'$) bound to 1 g. of gelatin are plotted against the experimental values of p_H. From these curves have been graphically derived the values of b' corresponding to equal increments of p_H (Table I). The large number of experimental points reduces the danger of such a graphic analysis significantly distorting the data.

Since pepsin does not carry the digestion of protein beyond the stage of complex polypeptides we are entitled to assume that, in the hydrolytic products, the original ionising groups are still separated from one another and from the extra liberated groups by relatively great molecular distances.

Table I.

Digest mixture 0·32 % gelatin-nitrogen, 0·029 % pepsin-nitrogen } p_H 2·4.

$b' \times 10^5$ per g. gelatin

	Total				Increment		
		Digest			Digest		
p_H	Control 24 hr	24 hr	48 hr	7 day	24 hr	48 hr	7 day
2·0	− 117·0	− 145·5	− 153·5	− 173·0	− 28·5	− 36·5	− 56·0
2·4	− 109·0	− 137·0	− 146·0	− 164·0	− 28·0	− 37·0	− 55·0
2·8	− 99·0	− 123·0	− 131·5	− 147·0	− 24·0	− 32·5	− 48·0
3·2	− 85·5	− 103·5	− 109·5	− 123·0	− 18·0	− 24·0	− 37·5
3·6	− 65·5	− 80·0	− 83·0	− 94·0	− 14·5	− 17·5	− 28·5
4·0	− 45·5	− 54·0	− 56·0	− 64·5	− 8·5	− 10·5	− 19·0
4·4	− 25·0	− 30·0	− 31·0	− 37·5	− 5·0	− 6·0	− 12·5
4·8	− 7·5	− 8·5	− 10·0	− 14·5	− 1·0	− 2·5	− 7·0
5·2	+ 5·0	+ 6·5	+ 4·5	+ 1·5	+ 1·5	− 0·5	− 3·5
5·6	14·0	16·0	15·0	10·5	+ 2·0	+ 1·0	− 3·5
6·0	19·5	23·0	22·5	18·5	3·5	3·0	− 1·0
6·4	24·0	29·5	29·0	26·0	5·5	5·0	+ 2·0
6·8	28·0	37·0	38·0	37·0	9·0	10·0	9·0
7·2	32·0	47·0	48·5	49·5	15·0	16·5	17·5
7·6	36·5	57·0	59·5	63·5	20·5	23·0	27·0
8·0	41·0	65·5	70·0	76·0	24·5	29·0	35·0
8·4	45·5	72·0	77·5	87·0	26·5	32·0	41·5
8·8	50·0	78·5	85·0	97·5	28·5	35·0	47·5
9·2	56·0	86·0	92·0	106·5	30·0	36·0	50·5
9·6	63·5	94·5	100·5	115·5	31·0	37·0	52·0
10·0	72·0	104·0	110·0	126·0	32·0	38·0	54·0
10·4	80·5	112·5	119·0	135·0	32·0	38·5	54·5
10·8	84·0	116·5	122·5	138·5	32·5	38·5	54·5

Consequently, their "strengths" should not be profoundly modified by the appearance of these new groups. Provided, therefore, there that has been no simultaneous condensation of free groups, we should be able to arrive at an approximation not only to the amounts but also to the "strengths" of the liberated groups by subtracting from one another the curves of the protein before and after digestion. This has been done in Table I. The resultant figures have, in one case (7 days digest), been charted as discrete points in Fig. 2. The continuous curve which has been superimposed upon these points is the theoretical curve for an ampholyte having $p_{K'}$ values of 3·5 and 7·5 and a concentration equal to the increment in equivalents of base bound by the protein at p_H 10. These $p_{K'}$ values proved to be the best for all titrations conducted at 38°. In other experiments, in which titrations were carried out at 25°, it was found that the best fit was obtained by assuming constants of 3·5 and 7·9. This shift of $p_{K_2'}$ with temperature may be taken to be interesting confirmation of the conclusion that the liberated groups which ionise in this region are basic groups since the two values which have been quoted both correspond to a p_{k_b} value of 6·0.

The data which have been presented seem to establish quite definitely that the ratio of the increments in acid- and in base-binding capacities of gelatin closely approximates to unity at each stage in the digestion. Moreover, it is suggested that the mean dissociation constants of the acidic and basic

groups set free are closely represented by $p_{K_1'} = 3{\cdot}5$ and $p_{K_2'} = 7{\cdot}9$ (at 25°) respectively. Since all experiments led to the same unequivocal result, it may be taken to be independent of the composition of the digest within the limits stated at the outset. It remains to compare the mean dissociation

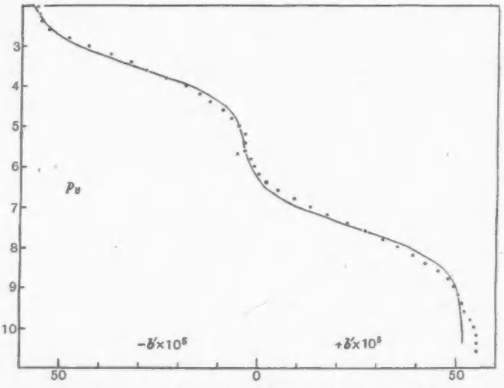

Fig. 2. "Titration curve" of groups liberated in peptic digestion. The continuous curve is the theoretical curve for an ampholyte having $p_{K'}$ values of 3·5 and of 7·5 (at 38°). The corresponding values at 25° were found to be 3·5 and 7·9.

constants which have been derived with those of the -COOH and $-NH_2$ groups of peptides of increasing complexity to arrive at the reasonable inference that the main chemical effect of pepsin is the hydrolysis of peptide unions between α-COOH and α-NH_2 groups of amino-acids (Table II).

Table II. *Dissociation constants of peptides* (from Kirk and Schmidt [1929]).

	p_{K_1}	p_{K_2}
Glycine	2·37	9·72
Alanine	2·35	9·72
Glycylglycine	3·12	8·07
Glycylalanine	3·15	7·9
Glycylglycylglycine	3·26	7·9
Glycylalanylalanylglycine ...	3·30	7·9
Groups set free by pepsin (cf. text)	3·5	7·9

Apart from this, the major effect of pepsin, two minor effects are suggested by our results. One of these is the progressive displacement of the curve in the isoelectric region as digestion proceeds (Fig. 1). The other is the indication of a small increase in base-binding capacity in the zone p_H 9–10 (Fig. 2). These effects were constantly observed and were absent from the curves of control mixtures. Their magnitude—less than 5×10^{-5} equivalents per g. gelatin even after 7 days—is, however, scarcely greater than the cumulative errors which are involved in our treatment of the data. If we accept 61,000 as the molecular weight of gelatin [Kunitz, 1926–7] it may be calculated that such an increase in b' corresponds to the formation of only two new ionising

groups per molecule. That is to say, our argument does not claim to be able to demonstrate an effect which is restricted to the hydrolysis of only a single protein linkage. At the same time, we may say that the two effects which have been referred to must, if they are real, be quite restricted in their incidence upon the protein molecule.

In conclusion, it should be pointed out that we have chosen to compare the titration curves of all digest mixtures with the initial curve for the control mixture rather than with that of a control of the same age. This has been done merely to permit economy in the reproduction of data. It is justified by the fact that the small changes which do occur in the controls indicate that the small amount of hydrolysis occurring under the influence of the acid present liberates groups of a character similar to those set free in much greater amount by the pepsin.

The relation between the chemical and physical changes in peptic digestion.

Comment was made, in the introduction, on the remarkable physical changes which are found to accompany relatively small increases in titratable groups. This may suitably be illustrated by an experiment in which only a low concentration of pepsin was employed. The viscosity of the mixture fell to one-half its original value in only 90 minutes. Moreover, the time of gelation increased rapidly during the first 4 hours. At the end of 6 hours the digest failed to form a gel at the isoelectric point at 0°. We may, therefore, conclude that unchanged gelatin rapidly disappeared. Within the same period the amount of nitrogen soluble in 80 % alcohol at the isoelectric point (in water) increased rapidly. Only after much longer periods was it possible to demonstrate important increases in titratable groups. The pertinent data are assembled in Table III.

Table III.

Digestion mixture 0·35 % gelatin-nitrogen, 0·003 % pepsin-nitrogen } p_H 1·9.

Equiv. × 10^5 per g. gelatin

Digestion time (hours)	Alcohol-soluble nitrogen	Acid bound		Alc. sol. N / NH_2 set free	NH_2 groups set free per mol.	Hydrolysis %
		Total	Set free			
Zero	10	77	—	—	—	—
2	52	—	—	—	—	—
4·5	75	—	—	—	—	—
9	109	84	7	14	4·3	0·7
22	176	88	11	15	6·8	1·1
70	304	96	19	15	11·7	1·9
168	361	102	25	14	15·4	2·4
356	420	107	30	14	18·5	3·0

The last column gives a figure for the percentage of the total hydrolysis which had been accomplished. This is the percentage of the peptide nitrogen of gelatin which is represented by the NH_2-nitrogen set free. The peptide nitrogen was calculated from the total nitrogen and from Dakin's figures [1920] for the composition of gelatin. The assumption has been made—a necessary

one on the basis of the polypeptide theory of protein structure—that proline and hydroxyproline can form peptide-like unions on their nitrogen atoms. The sixth column gives the number of peptide linkages split per molecule on the assumption that gelatin has a molecular weight of 61,000.

An interesting relation is disclosed by column 5. It will be seen that each peptide linkage hydrolysed rendered soluble in 80 % alcohol 14–15 atoms of nitrogen. A ratio of similar magnitude was found in all experiments in which it was sought provided hydrolysis had not proceeded beyond about 7 % of complete digestion. What significance may be attached to this relation is uncertain in view of the empirical nature of fractionations effected by precipitation with alcohol. It does, however, suggest that the fragments resulting from the early peptic digestion of gelatin are of large mean molecular weight.

We hope to be able to continue the investigation of some of the questions which have been raised.

Summary.

1. The changes in acid- and in base-binding capacities which accompany the digestion of gelatin by pepsin have been determined with the aid of hydrogen electrode titration curves. The results indicate that the main chemical effect of pepsin is the hydrolysis of peptide linkages constituted between the α-amino- and α-carboxyl-groups of amino-acids.

2. Some observations are added correlating the chemical changes with certain changes in the physical behaviour of the protein.

Grateful acknowledgement is made to Prof. Victor Myers for extending to one of us (R. K. C.) the hospitality of his department, and for his interest in the work here reported. The same author is indebted to the Medical Research Council for a grant in support of his work.

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CXV. THE CHEMISTRY OF OESTRIN.
IV. THE CHEMICAL NATURE OF CRYSTALLINE PREPARATIONS.

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Introduction.

During the last few months several claims have been made to have isolated the oestrus-producing hormone in a crystalline form. Owing to the different methods of assay employed by different workers, it is difficult to make a comparison of these preparations on the basis of their physiological activity. In the opinion of the author differences in the technique of biological assay may result in apparent variations in potency of several hundred per cent in a single preparation. In order to decide therefore the identity or otherwise of these preparations, chemical and physical data must be relied on.

Wieland, Straub and Dorfmüller [1929] described the preparation of active crystalline material melting at 154–165°, finally becoming liquid at 210°. As the authors themselves suggest, the material was probably not a single chemical substance. The activity of the preparation tested in aqueous solution by giving five injections over a period of about 48 hours was as low as 2,000,000 mouse units per g. This is probably significantly lower than the potency of some more recent preparations.

Butenandt [1929] prepared an active crystalline substance, which from the constancy of its activity after recrystallisation and resublimation he believed to be the hormone itself. The substance melted at 240° with decomposition, and molecular weight determinations and analyses pointed to the molecular formula $C_{23}H_{28}O_3$ or $C_{24}H_{32}O_3$. It was found to be unsaturated. On account of its behaviour with aqueous alkali, he suggested that the substance was a hydroxylactone. In a later paper [Butenandt and Ziegner, 1930] the melting point is given as 243°.

Shortly afterwards Dingemanse *et al.* [1930] described what appeared to be the same substance, judging from the melting point and analytical figures. There was some difference in the apparent activity of the two preparations, but this may possibly be accounted for by the fact that Butenandt injected an oily solution of the hormone [Butenandt and Ziegner, 1930]. This point has been discussed in a previous paper [Marrian, 1930]. Dingemanse *et al.* are not yet satisfied that their substance is the pure hormone, since several of their preparations had a significantly higher potency than others.

Doisy, Veler and Thayer [1930] have recently prepared what they claim to be the hormone itself in crystalline form. The crystals showed a constant activity and melting point after as many as twenty recrystallisations from several different solvents. In a later paper [Thayer, Veler and Doisy, 1930], evidence that the substance has the molecular formula $C_{18}H_{21}(OH)_2$ is put forward[1]. Iodine value determinations showed that one double bond was present. The melting point (243° uncorr., 249° corr.) suggests that this substance may be identical with those of Butenandt, and of Dingemanse *et al.* The published combustion figures for these two latter preparations do not differ widely from those required for $C_{18}H_{23}O_2$. It seems possible, therefore, that Butenandt may have been wrong in suggesting that his substance was a hydroxylactone of the formula $C_{23}H_{28}O_3$ or $C_{24}H_{32}O_3$.

In a further communication [Veler, Thayer and Doisy, 1930], they suggest, as other workers have previously done, that the hormone is weakly acidic, and they describe their method of isolation in greater detail.

In a previous paper in this series [Marrian, 1930] the isolation of an active crystalline substance was described. As far as could be judged by a microscopical examination of the crystals, it seemed to consist of a single chemical substance. Preliminary analyses and molecular weight determinations suggested the formula $C_{18}H_{24}O_3$. Since, however, the melting point was indistinct although considerably higher than that of any other preparation (256–261°), and since the crystals were slightly pigmented, no great confidence was placed in this formula. The smallness of the amount of material available at the time made further purification impracticable.

A further batch of the active crystalline substance has since been prepared by the same methods. By using ethyl acetate for the final recrystallisation instead of aqueous methyl alcohol, the crystals were obtained in what was obviously a much purer state. The melting point was higher and sharper than that of the original preparation (264–266° uncorr.), while analyses and molecular weight determinations confirmed the originally suggested molecular formula $C_{18}H_{24}O_3$.

The substance had weak but quite definite acidic properties. It was slowly but completely soluble in cold aqueous alkali and was precipitated from such a solution by carbon dioxide. It was quite insoluble in aqueous Na_2CO_3 solutions.

By treatment with hot acetic anhydride a crystalline acetate was obtained. From the weight of the crude acetate, analyses and molecular weight determinations, it was evident that three hydroxyl groups had been acetylated. Since this accounted for all the oxygen present, the acidic properties of the substance were evidently due to one or more phenolic hydroxyl groups. The acetate itself was quite insoluble in cold aqueous alkali, thus confirming the conclusion that the acidic group or groups had been acetylated.

[1] This is the formula put forward by Thayer, Veler and Doisy, but their results would agree equally well with the more probable formulae $C_{18}H_{20}(OH)_2$ or $C_{18}H_{22}(OH)_2$.

A hydrogen electrode titration showed that one molecule of the substance combined with only one equivalent of base. Only one acidic hydroxyl group was therefore present.

The possible relationship between this substance and other crystalline preparations is discussed in a later section of this paper.

Experimental.

Isolation of the active substance.

The methods of extraction of the hormone from the urine and subsequent methods of purification previously described [Marrian, 1930] were closely followed.

245 litres of urine were acidified and extracted with ether. The ether extract contained 4,600,000 mouse units, corresponding to a yield of 18,800 units per litre. The dried extract was heated with aqueous KOH, and carbon dioxide passed into the mixture for 12 hours, which was then extracted thoroughly with ether. The ether extract was evaporated to dryness and extracted with ice-cold acetone. The acetone extract, containing the hormone, was dried and extracted with ice-cold 50 % alcohol. The alcoholic extract was evaporated to dryness, dissolved in ether after the addition of a small amount of alcohol and extracted with aqueous KOH. After acidification of this alkaline extract, the hormone was extracted with ether.

This concentrate was dissolved in a small amount of alcohol and, after the addition of a large volume of redistilled ether, cooled to − 15°. A large amount of solid material separated and was filtered off. This solid was then boiled in alcoholic solution with charcoal, filtered and evaporated to dryness. On crystallisation of the residue from ethyl acetate, 0·181 g. of white crystalline material was obtained. These crystals melted at 264–266° (uncorr.) with very slight darkening, there being also a slight preliminary shrinkage and darkening at 255°.

Two assays of the potency of this substance gave values of 7,930,000 and 7,460,000 mouse units per g. The total amount of active material in crystalline form was therefore about 1,490,000 mouse units, corresponding to a yield of over 32 % of the activity of the original ether extract.

Solubilities and general properties.

The substance was characterised by a fairly low solubility in most of the common organic fat solvents. In ether the solubility was very low, in methyl and ethyl alcohols, chloroform and acetone it was somewhat greater. It was easily soluble, however, in pyridine.

In 5 % aqueous KOH it dissolved slowly but completely in the cold. On passing carbon dioxide into this solution, it was immediately precipitated. The melting point of this precipitated material was 263–265°, and its physiological activity determined as 7,430,000 and 7,350,000 mouse units per g. by duplicate assays.

In aqueous Na_2CO_3 solution it was quite insoluble.

Determinations of the iodine value by the method of Rosenmund and Kuhnhern using a solution of bromine in pyridine sulphate gave figures which at first suggested the presence of two double bonds. When the phenolic nature of the compound was demonstrated, it was realised that part of the bromine uptake might have been due to bromination of the phenyl radical. By this time insufficient material remained to determine the degree of unsaturation by other methods.

Wieland, Straub and Dorfmüller [1929] observed that their crude crystalline preparation gave an orange colour with a green fluorescence with the Liebermann-Burchardt reaction. A similar colour reaction was noted by the author [Marrian, 1930].

It has now been found that the crystalline preparation behaves in the same way merely on warming with concentrated H_2SO_4. The presence of chloroform and acetic anhydride seems to be unnecessary. The reaction was shown strongly by 0·02 mg. The colour given is indistinguishable from that given by crude bile acids under the same conditions.

Lipschutz's so-called "oxycholesterol" reaction with benzoyl peroxide, acetic and sulphuric acids gave a delicate rose-pink colour with 0·02 mg. of the substance.

Since the substance is probably unsaturated, Rosenheim's [1929] trichloroacetic acid test was tried. It was entirely negative on about 0·5 mg.

Optical rotation.

The rotation was determined in pyridine solution in a Fischer micro-tube using the mercury green line. In order to minimise personal errors, readings were taken by two independent observers.

8·1 mg. of the crystalline substance were dissolved in 0·3 cc. of dry, redistilled pyridine.

	C. R. H.	G. F. M.
Observed rotation =	+ 0·51°	+ 0·52°
$[\alpha]_{5461}$ =	+ 37·7°	+ 38·5°.

Analyses and molecular weight.

Micro-combustions and molecular weight determinations by Rast's camphor method gave the following results:

4·613 mg. gave 12·655 mg. CO_2 and 3·51 mg. H_2O.

C = 74·82 %, H = 8·51 %.

4·437 mg. gave 12·145 mg. CO_2, and 3·29 mg. H_2O.

C = 74·67 %, H = 8·30 %.

3·242 mg. gave 8·890 mg. CO_2, and 2·42 mg. H_2O.

C = 74·79 %, H = 8·36 %.

Mol. w. = 286; 268 (Rast.).

Calculated for $C_{18}H_{24}O_3$:

C = 75·00 %, H = 8·33 %.

Mol. wt. = 288.

These results can leave little doubt that the substance examined is represented by the molecular formula $C_{18}H_{24}O_3$.

Preparation and properties of the acetate.

The general solubilities of the substance suggested the presence of hydroxyl groups. An attempt was therefore made to prepare an acetate. 21·2 mg. were heated to 110° for 2 hours with 2 cc. acetic anhydride. Excess of water was added and after scratching with a glass rod for some time a white solid separated. This was filtered off and was washed repeatedly with water. The crude acetate was then dissolved in hot alcohol and the alcoholic solution evaporated to dryness in a vacuum desiccator over solid KOH. The residue, which was a clear gum, was twice redissolved in alcohol and evaporated over KOH to ensure the complete removal of acetic anhydride.

32·2 mg. of crude acetate were obtained. If three hydroxyl groups had been acetylated, the theoretical weight of acetate should be 30·4 mg.

This crude acetate was dissolved in 1 cc. of hot methyl alcohol and hot water added drop by drop until a faint permanent cloudiness was produced. After cooling for some hours at 0°, rosettes of white needles were deposited. The crystals were filtered off, dissolved in a small volume of alcohol and reprecipitated by the addition of a large volume of water.

The melting point of this preparation was 117·5–119°. A later preparation melted at 120–122°.

Unlike the parent substance, the acetate was quite insoluble in aqueous KOH.

The physiological activity of a specimen of acetate (M.P. 120–122°) was 3,740,000 mouse units per g. On the basis of the amount of original substance present in the acetate, the activity corresponds to 5,370,000 mouse units per g. Thus the conversion of the substance to its acetate appeared to have decreased its physiological activity to a significant extent.

Analysis and molecular weight of the acetate (M.P. 117·5–119°).

Analyses and molecular weight determinations clearly confirmed the view that three hydroxyl groups had been acetylated:

4·965 mg. gave 12·625 mg. CO_2 and 3·23 mg. H_2O.

C = 69·35 %, H = 7·28 %.

4·699 mg. gave 11·950 mg. CO_2 and 3·09 mg. H_2O.

C = 69·35 %, H = 7·36 %.

Mol. wt. = 377; 402 (Rast).

Calculated for:

$C_{18}H_{23}O_2 . OCOCH_3$,	C = 72·73 %,	H = 7·88 %,	mol. wt. = 330.
$C_{18}H_{22}O . (OCOCH_3)_2$,	C = 70·96 %,	H = 7·53 %,	mol. wt. = 372.
$C_{18}H_{21} . (OCOCH_3)_3$,	C = 69·56 %,	H = 7·25 %,	mol. wt. = 414.

Hydrolysis of the acetate.

0·0315 g. of acetate (M.P. 120–122°) was heated for 1 hour to 100° with alcoholic sodium ethoxide in an atmosphere of nitrogen. Excess of water was added and the mixture saturated with carbon dioxide, when a white solid separated. This was filtered off, washed repeatedly with water, dried and recrystallised from ethyl acetate. This substance melted at 267–268° with decomposition. Its physiological activity was found to be 7,400,000 mouse units per g. It seems certain therefore that saponification of the acetate yielded the original substance again.

Hydrogen electrode titration of the active substance.

The data presented in the preceding sections show clearly that the active compound is a trihydroxy-substance and that the acidic properties are due to the presence of one or more acidic hydroxyl groups. It was obviously impossible to determine the number of the acidic groups by direct titration. Mr Kekwick and Mr Cannan therefore very kindly undertook to carry out a hydrogen electrode titration.

The electrode technique was that described by Cannan and Knight [1927]. 20 cc. of a 0·01 *M* solution of the active compound in 0·1 *M* NaOH were titrated with 0·1 *M* HCl. The solution remained free from precipitate throughout the greater part of the titration during which the p_H range covered was 12·7–11·5. Calculation showed that the base bound by the substance within this range was constant and corresponded to an equivalent weight of 268–298. At p_H 11·5 precipitation commenced and simultaneously the amount of base bound to the substance progressively diminished, the curve indicating the presence of a group influencing ionisation within the p_H range 9–11·5. The curve was, however, distorted by the presence of the solid phase. It was assumed that this was due to the low solubility of the undissociated molecule.

A rough determination of the solubility of the substance in 0·1 *M* NaCl was made physiologically. A small quantity of the crystalline substance was shaken for 3 days with 0·1 *M* NaCl at the temperature of the experiment. The solid was allowed to settle and a small volume of the supernatant fluid pipetted off. The physiological activity of this was then determined in the usual manner. In this way it was shown that the solubility of the substance in 0·1 *M* NaCl was of the order of 0·002–0·003 %, *i.e.* approximately 0·0001 *M*.

Assuming that this value applied under the conditions of the experiment, appropriate corrections of the experimental titration curve were made. As a result a p_K of approximately 10·8 was deduced. Making allowances for the

uncertainties involved in the assumptions made, it may be stated that the p_K value is probably within the range 10·5–11·0. It may therefore be said with some certainty that one and one only of the three hydroxyl groups in the molecule is acidic.

Phenolic nature of the acidic hydroxyl group.

The magnitude of the approximate p_K is of the order of that of a phenolic group. However, further proof of the phenolic character of the substance was sought. Certain colour reactions characteristic of phenolic substances were therefore carried out on a colloidal aqueous suspension of the substance, made by adding water to an alcoholic solution.

Millon's reaction was strongly positive. With diazotised *p*-nitraniline[1] a stable purple colour resulted, which was indistinguishable from that given by tyrosine under the same conditions.

On warming some of the dry substance with strong HNO_3 (xanthoproteic reaction), the solid immediately turned a canary yellow, subsequently dissolving to form a yellow solution.

These reactions definitely show that a hydroxyphenyl radical is present in the molecule.

Attempted distillation of the active substance.

The preparations of Butenandt [1929] and of Dingemanse *et al.* [1930] apparently distilled under reduced pressure with some ease. The former preparation distilled at 130–150° and 0·02–0·03 mm. Hg, the latter at 130–150° and 0·01 mm. It was therefore of interest to see whether the compound under investigation would behave in the same way. The author wishes to express his gratitude to Mr Askew, who kindly carried out the distillation at the National Institute for Medical Research.

The experiment was performed on 20 mg. of the crystalline substance, representing about 154,000 mouse units, at a pressure of approximately 0·001 mm. Hg.

The temperature was first raised to 146° for about 45 minutes. Since there was no visible distillate, the temperature was raised to 156° for 1 hour. At this temperature a small amount of material sublimed in the cooler part of the distillation tube, but the process was evidently so slow that the temperature was further raised to 165° for 1 hour. Only very slightly more material sublimed at this temperature. Since there appeared to be a slight degree of decomposition at this temperature, the experiment was abandoned.

The amount of material sublimed was so small that it was quite impossible to weigh it accurately or to determine its physiological activity. Since it melted at 260° it seemed that the original substance had sublimed unchanged.

The unsublimed residue weighed 19·6 mg. and melted at 261–263°. Its physiological activity was unchanged (8,330,000 mouse units per g.).

[1] Unpublished work by Harington and Shüpbach.

Discussion.

The outstanding problem is to decide the relationship between the active trihydroxy-substance described in this paper and the active dihydroxy-substance isolated by Thayer, Veler and Doisy, which may or may not be identical with that isolated by Butenandt.

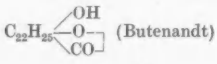
A possible explanation would be to assume that the trihydroxy-substance owes its activity to contamination with the substance melting at 243°. Putting the relative potency of the latter at the highest possible figure, it would then be necessary to suppose that the former was contaminated with at least 15–20 % of the latter. If this were the case it is difficult to explain the melting point of 264–266° of the author's substance.

The failure of the active compound to distil under conditions even more favourable than those in Butenandt's or Dingemanse's experiments makes it still more improbable that the volatile substance melting at 243° is present as an impurity.

On the other hand the constancy of the melting points of the substances described by these authors after frequent recrystallisations makes it impossible to believe that their substances are mixtures.

It is a curious fact that the acetate prepared by Thayer, Veler and Doisy has roughly the same melting point as that prepared by the author. The similarity may be a coincidence, but nevertheless, it is unfortunate that the former do not appear to have attempted to regenerate the original substance from it by saponification.

The only striking difference between the methods of isolation described in this and previous papers and those adopted by other authors, is that in the former, the extract is treated for some time with hot alkali. It is just possible that such treatment might result in a chemical change without seriously impairing the physiological activity. However, it is difficult to see how a substance of the formula



(Butenandt)

or $C_{18}H_{21}(OH)_2$ (Thayer, Veler and Doisy) could be changed in this way to a substance of the formula $C_{18}H_{21}(OH)_3$.

Alternatively, it could be supposed that two or even three substances originally present in the urine possess oestrus-producing activity. It seems unlikely, however, that oestrogenic activity could be an intrinsic property of three different molecules. A more probable explanation would be that none of these substances is the hormone itself, but rather that they are different inactive compounds, each associated with minute amounts of the real active principle. It is not at all inconceivable that the different methods of purification should result in the isolation of different substances.

The evidence that any of these substances is the pure hormone cannot yet be accepted as conclusive. The fact that the activity is the same for different

batches and is unchanged after recrystallisations is not definite proof. It will be recalled that frequent recrystallisations were found by many workers to be quite ineffective for the removal of provitamin D from cholesterol or phytosterol. The association of oestrogenic activity with the substances under discussion may possibly be found to be a similar problem.

On the other hand, it is curious that the author has so far failed to isolate any substance from urine with a melting point of 243°, while the other authors have not so far reported the isolation of the higher melting substance.

A fact which may be of some significance is that the substances isolated by Dingemanse *et al.*, Doisy, Veler and Thayer and by the author are all stated to exist in two crystalline forms—plates or leaflets, and needles.

It must be admitted that at present no explanation can be put forward to explain all the facts adequately.

Summary.

1. Of the total oestrus-producing material in the ether extract of acidified urine 32 % has been obtained as a pure crystalline substance.

2. This substance (M.P. 264–266°) is represented by the molecular formula $C_{18}H_{24}O_3$. It is strongly dextro-rotatory. $[\alpha]_{5461} = +38°$.

3. The substance is weakly acidic, being soluble in aqueous alkali. It is precipitated from alkaline solution by carbon dioxide.

4. An acetate (M.P. 120–122°) has been prepared. Analyses and molecular weight determinations show that three hydroxyl groups are acetylated. The acetate is insoluble in aqueous alkali.

5. Determination of the base-binding power and approximate p_K of the substance show that one hydroxyl group is acidic.

6. Certain colour reactions characteristic of phenolic substances are strongly positive. It is concluded that a hydroxyphenyl radical is present in the molecule.

7. The activities and melting-points of the material precipitated from alkaline solution by carbon dioxide and of the material obtained by saponification of the acetate are the same as those of the original substance.

8. The substance is not appreciably volatile at 165° and 0·001 mm. Hg.

9. The relationship between this active substance and the active substances described by other authors is discussed.

The author is again indebted to Prof. F. J. Browne of University College Hospital for providing facilities for the collection of urine. Sincere thanks are due to Prof. J. C. Drummond and to Dr C. R. Harington for their keen interest and frequent helpful advice.

The expenses of this work were defrayed by a grant from the Medical Research Council to whom the author wishes to express his gratitude.

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CXVI. PROPERTIES OF THE COMB-GROWTH-PROMOTING SUBSTANCE OBTAINED FROM TESTES AND URINE.

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A series of publications has recently appeared concerning the preparation of comb-growth-promoting substances from testes and urine [Moore and McGee, 1928; McGee, Juhn and Domm, 1928; Moore, Gallagher and Koch, 1929; Funk, Harrow and Lejwa, 1930; Freud *et al.* 1930]. Similar work has been carried on in these laboratories and a method has been published [Dodds, Greenwood and Gallimore, 1930] for the preparation of the hormone in a water-soluble form. A study of the existing literature yields very little information concerning the properties of the active substance. The workers first mentioned proved that the active principle could be extracted from testes by means of benzene and that it was soluble in alcohol and ether. It is also stated that the active substance could be extracted by means of chloroform.

It was shown by us that the benzene-soluble material withstood saponification with barium hydroxide, and that after the insoluble barium soaps are removed the active substance is present in aqueous solution. In this communication the properties of this water-soluble material have been still further studied.

As in the previous communication the potency of the material has been tested on capons.

I. Method of testing for activity.

The earliest attempt to use the male castrated fowl as a material for the demonstration of the activity of testis extracts was made by Pézard [1911] who obtained the assumption of male characters and behaviour in a capon by injections of extracts from the testes of a cryptorchid pig. McGee, Domm and Juhn have employed the capon in an extensive assay of extracts of bull testis, the reaction taken as indicative of a positive result being the increased growth of the comb and wattles.

In the young cock, two types of growth in the head furnishings are found. In the first few weeks from hatching they increase in size at a rate relative to

increase in size of the body as a whole. Some time later, however, the rate of growth is suddenly accelerated and the comb and wattles become intensely red owing to the development and extension of the sub-epithelial vascular system. At this time the comb, which has previously been smooth, shows a roughened surface due to the projection from the surface of innumerable red papillae. The point at which this heterogonic growth occurs has been shown to be coincident with the onset of spermatogenetic activity in the testis. It varies considerably not only in different breeds but between different individuals of the same breed.

The extent to which this phenomenon is dependent on the presence of the reproductive glands is shown by the effect of prepubertal castration. Such early castration leads to the complete suppression of the second growth phase, although the first type of growth is not affected. In a complete castrate the comb becomes dry, scaly and anaemic, although it grows at a rate comparable to body growth as a whole and reaches its maximum about 30 weeks after hatching.

It is found that this second type of growth can be induced in castrated birds by injection of suitable preparations of mammalian testis.

Before proceeding to a description of the technique involved in the present series of experiments it is desirable to point out briefly certain precautions to be taken into consideration when using castrated fowls for such experiments.

If the birds are used before they reach the end of their growth period, then it is legitimate to accept as positive only those individuals in which the comb-growth after injection is such that it is thrown well outside the range of variation in comb size of control castrates of the same age.

Where extensive growth of the comb occurs a regression ought to ensue following cessation of the injections.

Increase in comb size should be coincident with the development of red papillae from the surface of the comb. These first appear at the base of the comb and spread upwards until even the points of the comb show a strong development of them.

From a review of castrated birds in the stock used for experimental purposes it was found desirable to discard any birds which at maturity exhibited a comb length greater than 5 cm., since in almost every case such birds had not been completely castrated but retained minute fragments of testis insufficient in amount at the time of *post mortem* examination to increase materially the size of the comb beyond that of the complete castrate, but it is always possible that further development of the testicular fragment some considerable time later may produce an increased comb growth. Many of these birds, while possessing the same size of comb as a complete capon, may yet be recognised by the tendency, more or less well expressed, to develop red papillae.

Since in the following experiments only a qualitative and not a quantitative result was sought, and further since the amount of extract available for use in

any one experiment was limited, only those birds were used which as adults possessed a comb falling within the previously defined limit of variation for castrates of the brown Leghorn stock used [Dodds, Greenwood and Gallimore, 1930]. The birds were bred at the department and castrated in the first few weeks after hatching (3–78 days). The combs were measured at fortnightly intervals during the development of the birds, and since the operation had never shown any development of red papillae characteristic of testicular activity, but were dry and scaly. As mature birds they had shown no signs of growth for several weeks prior to the experiments.

The most satisfactory measurement of comb size would be a volumetric determination, but, while such a method has been evolved, the great care necessary to obtain an accurate result and the time involved makes it impracticable when dealing with numerous experimental individuals. Moreover, in the case of small increments in comb size the chance of error is great. McGee, Domm and Juhn used the product of length and breadth as an expression of comb size and obtained an index of growth by dividing the initial product into the length-height products obtained during the course of the experiment. This method was rejected in the present series of experiments in favour of a simple measure of maximum length as an indication of increase in comb size because in the first place only qualitative results were desired

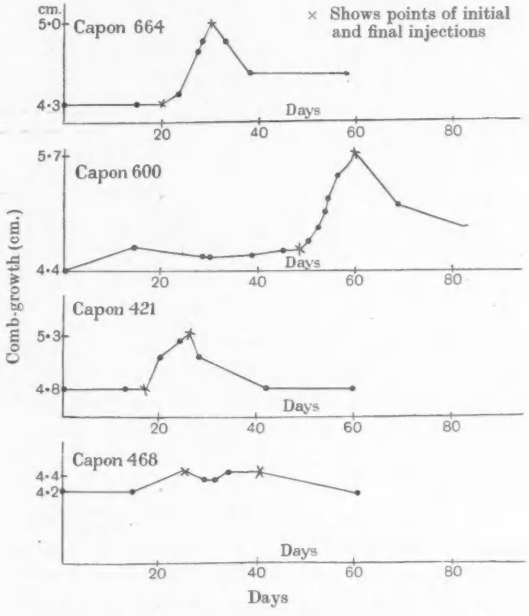

Fig. 1. Specimen charts showing record of comb-growth.

and it was found that increase in comb length was invariably accompanied by increases in height of the comb. Secondly, because, although two well-defined points are available for the accurate measurement of comb length, the measurement of height presents only one such well-defined point at the apex of the comb and an indefinite measuring point where the comb merges into the skin of the head. It was thought, therefore, that less error would be obtained where the single measurement was employed in view of the fact that relatively small increases in size were to be expected from the amount of extract at disposal for injection (Fig. 1).

The second criterion of activity of a given extract was the development of scarlet papillations on the otherwise dry scaly comb and the simultaneous sloughing off of the dry scales in these areas.

It was felt that any attempt at a statistical experiment to show the relationship between comb-growth and the amount injected had better be postponed until a highly active substance could be obtained. With crude extracts the rate of absorption, etc. might vary the response in a similar manner to the effects of crude oily preparations of the oestrus-producing hormone on rats.

II. Chemical properties.

The following experiments were designed to show the general properties of the active substance as prepared by the method already described [Dodds, Greenwood and Gallimore, 1930]. The extract used was water-soluble and prepared by the barium hydroxide method of hydrolysis either from bulls' testes or from concentrated male urine (solution A). In each experiment the starting material was divided into two portions, one of which was subjected to the treatment described, whilst the other was used as a control. In the case of the enzyme experiments the control was treated with a boiled solution of the enzyme. Both of the final solutions were injected into at least two capons and the result was recorded as to whether a definite sudden increase in comb-growth was produced. In all the experiments recorded the control gave positive results.

The action of mineral acids.

Sufficient HCl was added to solution A to make the concentration $N/10$. The resulting solution was boiled for 1 hour under reflux, cooled and neutralised. The control of solution A was boiled for 1 hour in neutral solution. Injection into the capon proved that the activity was not destroyed.

The action of trypsin.

An active solution of trypsin was prepared of which 0·2 cc. digested 10 cc. of 0·1 % alkaline caseinogen solution in 40 minutes (method of Gross, 1907). Two portions of 50 cc. each of solution A were taken and the reaction of both was adjusted so that it was just alkaline to phenolphthalein. One portion was incubated for 2 hours at 40° with 10 cc. of the active trypsin solution, whilst

the control quantity of 50 cc. was incubated with 10 cc. of the same trypsin solution which had been previously boiled for a sufficient length of time to destroy its activity. After the incubation both solutions were neutralised. The test showed that the potency was destroyed by trypsin under these conditions.

The action of pepsin.

An active solution of pepsin was prepared of such a strength that 0·005 cc. just digested 2 cc. of 0·1 % edestin solution in 30 minutes (method of Fuld, 1907). Two quantities of solution A were treated with the pepsin solution; in one case the activity had been destroyed by boiling in exactly the same manner as described above, with the exception that the reaction was adjusted to the acid side of Congo red. On injection it was found that the activity was partially destroyed.

The action of oxidising agents.

Hydrogen peroxide was added to solution A in sufficient concentration to render it 1·7 *N* (estimated by permanganate titration). The solution was shaken vigorously for 1 hour and the excess of hydrogen peroxide was removed by boiling with sodium hydroxide. The control amount of solution A was treated with the same quantity of sodium hydroxide and was boiled, but no hydrogen peroxide was added. Injection showed that the activity was destroyed.

The solubility of the hormone in alcohol, benzene and light petroleum.

A series of experiments was performed in which attempts were made to extract the hormone from its solution by solvents. The result is expressed in Table I. From this it can be seen that the hormone is freely soluble in benzene, and that the benzene solution may be washed with *N* sodium carbonate, *N* HCl and 10 % potassium hydroxide solutions without any loss of potency in so far as it is possible to tell by the method of testing. The weight is considerably reduced by this treatment. Also, when dissolved in 50 % alcohol the active substance can be completely extracted with light petroleum.

Table I.

Urine 30 litres
| Evaporated to about 2 litres
| Acidified and extracted with benzene in the warm
Benzene-soluble 5500 mg.
| Benzene solution washed with *N* Na_2CO_3 solution
Benzene-soluble 255 mg. — Na_2CO_3 solution
| Dissolved in absolute alcohol, water added to make the alcohol 50 %, and extracted with light petroleum
Light petroleum-soluble 81 mg. — Alcohol-soluble
| Dissolved in benzene, washed with *N* HCl and then washed with 10 % KOH
Benzene-soluble 16 mg. — Alkali-soluble

All the extracts on the right of the table were inactive and the final product (16 mg.) when dissolved in sesame oil and injected into two capons for 10 days gave good comb-growth.

The above may be used as a basis for the preparation of the hormone.

Distillation of the active principle.

For these experiments a triple stage Gaede mercury-vapour pump was employed. The distillation apparatus was made of glass in one piece and was jointed directly to the top of the pump (Fig. 2). The material to be distilled was poured into the bulb and after very careful drying over P_2O_5 was heated in an oil-bath. The side-tube was cooled with a freezing mixture. The vacuum obtained was less than 0·1 mm. It was found impossible to distil the crude benzene extract of testes or concentrated urine as excessive frothing occurred accompanied by the evolution of benzoic acid in the case of the latter. Successful results, however, were obtained with purified material. Solution A was evaporated to dryness and the residue was dissolved in alcohol. The alcoholic solution was transferred to the flask of the distillation apparatus and the alcohol was evaporated off. After careful drying distillation was carried out by the method described. Distillation began at 90° when a small amount of the material came over, but the greater part came over between 140° and 160°. The distillate was found to consist of a mixture of crystals and pale yellow oil and was readily soluble in water. On injection the material was found to be active. A similar distillation experiment was performed with the material purified by light petroleum extraction. It was found that the maximum distillation occurred between 140° and 160° and the distillate in this case consisted of a dark oil insoluble in water; it was dissolved in sesame oil and was found to be active. The temperature was raised to 200° and it was found that the residue was inactive.

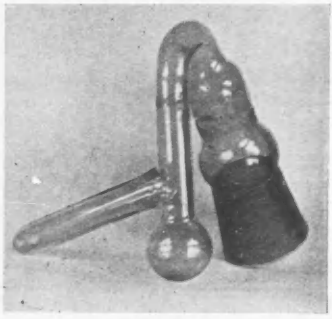

Fig. 2.

OTHER PHYSIOLOGICAL PROPERTIES.

Effect on seminal vesicles.

In these experiments rats and mice were used. Difficulty was experienced in obtaining consistent results, until it was realised that there is a great variation in the size of the seminal vesicles of normal rats. Moreover, the rate of atrophy of these organs after castration is very variable, and may in some cases be extremely slow. It has, therefore, been found advisable to allow a period of about 6 months to elapse after castration before using the animals

for testing purposes, and in addition we have found it advisable to perform exploratory laparotomy some few days prior to the actual test. In this way the seminal vesicles can be inspected and the animals destined for injections can be compared with the controls. Experiments on mice showed a similar variability in the size of the seminal vesicles and in the rate of atrophy after castration.

The difficulty in regard to the slow rate of atrophy can be avoided by operating on the animals before the vesicles have reached any considerable size. Since rats and mice may be operated on at 3 weeks of age without any appreciable operative mortality, suitable material for the test may easily be obtained. It would appear that very young animals require more of the extract to bring about observable growth of the seminal vesicles than fully grown ones. This may be comparable to the fact observed by Parkes and Bellerby [1926] that the new-born female rat or mouse does not respond to oestrin.

Table II.

Animal	No. of injected animals	No. of control animals	Age	Time interval between experiments and castration	Injections	Result on seminal vesicles
Rat	3	1	—	5 months	Twice daily for 5 days	Positive
Rat	4	2	16 days	Not castrated	Twice daily for 4 days	Negative
Rat	3	1	—	7 months	Twice daily for 6 days	Positive (Fig. 3*a*)
Rat	3	1	—	? months	Once daily for 15 days	Strongly positive
Mouse	4	2	4 weeks	1 day	Twice daily for 8 days	Negative
Mouse	4	2	4 weeks	9 days	Once daily for 2 days	Negative
Mouse	1	1	6 weeks	2 weeks	Once daily for 10 days	Strongly positive
	3				Once daily for 22 days	,, (Fig. 3*b*)
Mouse	4	1	28 days	5 days	Twice daily for 4 days	Positive

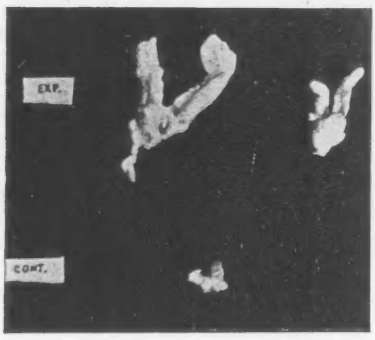

Fig. 3*a*.

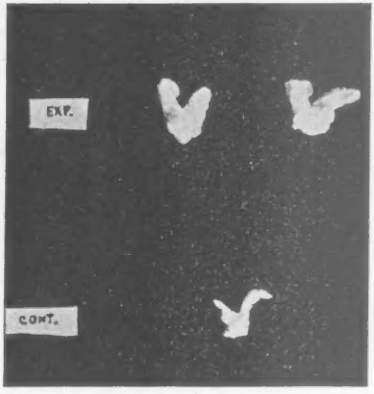

Fig. 3*b*.

The extracts from testes do not produce oestrus when injected into ovariectomised rats.

SUMMARY.

1. The preparation and properties of a comb-growth-promoting factor, and a qualitative examination of its activity on capons, are described.

2. The activity of this factor is destroyed by pepsin, trypsin and oxidation, but survives boiling with acids and alkalis.

3. The benzene solution containing the active substance can be washed with strong acids and alkalis, and the active substance can be extracted from a 50 % alcohol-water solution with light petroleum. These properties have been utilised in a provisional method for the extraction and purification of the active principle from male urine.

4. The hormone can be distilled in a high vacuum, but only after previous purification and drying.

5. When injected into male castrated rats or mice the atrophied seminal vesicles regain their normal size. The extracts from testes do not produce oestrus.

It is a pleasure to thank Mr Edwards, of Messrs W. Edwards & Co., Ltd., London, S.E. 5, for the loan of the vacuum pump and for his advice with regard to the necessary apparatus.

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CXVII. ON THE MECHANISM OF SUGAR EXCRETION.

I. GLUCOSE.

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When the blood-sugar is raised and sugar is excreted in the urine, this excretion is, according to the secretion theory, due to a stimulation of the tubule cells by the increased sugar percentage. In answer to this stimulation the tubule cells secrete sugar into the urine. The filtration-reabsorption theory, on the other hand, holds that sugar normally filters out through the glomeruli, but that all that is filtered is, under normal conditions, reabsorbed. When the percentage of sugar in the blood is raised, the tubule cells fail to reabsorb all; some of the sugar is left in the urine and is excreted.

It was part of our plan to use the experiments reported in this paper to contribute to an answer as to which of the two theories is right. Our viewpoint was that it would perhaps be possible to demonstrate that the kidney during prolonged sugar-excretion became fatigued. If so, the result of a fatigued secretion should be different from that of a fatigued reabsorption. If the mechanism of sugar-excretion is a secretion by the tubule cells and if the cells responsible for the secretion could become fatigued, one might expect that the excretion of sugar after repeated injections of sugar would diminish. If, on the other hand, sugar is excreted as postulated by the filtration-reabsorption theory the cells which could become fatigued would be those responsible for the reabsorption. Fatigue should show itself in failure of reabsorption, that is, increased excretion after the second injection.

While our experiments cannot contribute to this part of the problem because—as will be shown later—no definite difference could be demonstrated in the sugar-excretion after repeated sugar injections, experiments have been made by another author which answer the question.

Poulsson [1930] has made a series of experiments in which he studies phloridzin-glycosuria by means of the creatinine method [Rehberg, 1926]. The result of the experiments is, briefly, that the amount of sugar excreted in a completely phloridzinised dog corresponds closely to the amount which should be excreted according to the calculations of filtrate made by the creatinine method.

The amount of blood cleared of sugar is for each minute nearly the same as the amount cleared of creatinine.

While this fact is in complete accordance with the filtration-reabsorption theory and only needs the assumption that phloridzin abolishes the sugar reabsorption, it is hard to explain on the basis of the secretion theory. It makes it necessary to assume that under phloridzin poisoning the tubules begin to secrete sugar and moreover do so at exactly the same rate as that at which they secrete creatinine—an assumption which is possible but certainly not likely.

We think that the experiments of Poulsson together with what is further known about the excretion of sugar—especially the presence of sugar in the glomerular filtrate and its absence from the urine of the frog [Richards, 1929]—practically proves that sugar is actually filtered and not secreted, a fact which once accepted leads of necessity to the conclusion that the whole theory is in the main right.

The other part of our programme was to study how the glycosuria arising out of a too high sugar level in the blood should be looked upon according to the filtration-reabsorption theory, if this was once accepted. Our experiments may be regarded as a supplement to those of Poulsson, which were concerned with the mechanism of phloridzin glycosuria, while ours are concerned with the mechanism of normal glycosuria.

The explanation offered for glycosuria in the normal state is that any excess above the normal percentage present in the glomerular filtrate fails to be taken up by the tubule cells and passes into the urine, but how we are to look upon this failure is not clear.

Different possibilities can be conceived. (1) A regulation might exist, whereby the reabsorption of sugar was either stopped completely or decreased, when the blood-sugar was increased above a certain level. (2) The reabsorption could go on at unchanged rate, thus leaving the excess of sugar to be excreted. (3) The reabsorption could increase with the blood-sugar, but fail to follow the increase completely and in this way leave some sugar to be excreted.

To ascertain which of these possibilities was responsible for the excretion of sugar was the further aim of our experiments.

The plan of our experiments was to use the creatinine excretion as an index for the filtration rate [Rehberg, 1926].

That creatinine really is an indicator of the filtration rate is made highly probable by the following facts.

(1) Creatinine is the substance which is concentrated most by the kidney. In man the concentration ratio $\left(\frac{\text{percentage in urine}}{\text{percentage in blood}}\right)$ can reach 400. More blood is cleared of creatinine than of any other substance.

(2) Creatinine excretion is independent of urine volume, which proves that nothing diffuses back or is reabsorbed in the tubules.

(3) Creatinine excretion seems to be dependent on the osmotic pressure of the blood colloids in such a way that the excretion decreases with increasing osmotic pressure of the blood colloids [Ni and Rehberg, 1930]. This is easily

understood if creatinine is excreted by a filtration process but hard to explain from the secretion standpoint.

We therefore think it justifiable to postulate that the amount of filtrate formed in the glomeruli per minute must be the same as the amount of blood cleared of creatinine per minute, and from this view-point our experiments have been planned.

The experiments were made on a dog in which ureter-fistulae had been made after the method of Orbeli[1]. The operation was made in September and the dog recovered well from it and was in good health during the experiments. It was well trained and accustomed to the experimental conditions.

The experiments were made according to the following plan. The dog was fed in the afternoon and received no food from the evening before an experiment, but was allowed plenty of water for the night. At the beginning of an experiment an intravenous injection of a varying amount of glucose was given together with usually 1 g. of creatinine. The glucose used was dissolved the day before, made alkaline and kept for about 18 hours at 20°. Before injection it was neutralised. The amount of water in which the sugar was dissolved was varied. About 5 minutes after the injection the collection of urine was started —the urine from both ureters being collected together through a common funnel fastened over the ureter openings. Urine was collected in periods, the lengths of which were varied according to the magnitude of the diuresis.

At intervals during the experiment blood was drawn from the saphenous vein. The blood was kept from clotting by a small amount of lithium oxalate. Both urine and blood samples were cooled down immediately after taking and the blood was kept on ice until centrifuging took place.

Plasma was used for the determinations of sugar and creatinine.

For the calculation of the rate of the glomerular filtration and the estimation of creatinine in the samples of plasma and of urine, the method described in another paper [Rehberg, 1929] was followed. Control experiments showed that glucose added to urine up to 1200 mg. per 100 cc. did not change appreciably the creatinine readings taken between 5 to 100 minutes after the addition of sodium hydroxide. The urine was therefore diluted so that the sugar concentration was less than 1 % and the readings made as soon as possible.

Urinary glucose was estimated by polarisation. The polariscope[2] was a Schmidt and Haensch instrument which could be read to 0·01°. The urine was acidified with acetic acid and cleared by grinding with lead acetate and filtering.

The error caused by this treatment was found to be within the sensitivity of the polariscope. Tubes of 2 dm. length were used. Control experiments showed that fasting urine under various degrees of diuresis did not show any

[1] The operation was kindly performed by Prof. Orbeli himself during a visit to our laboratory and we hereby beg him to accept our best thanks for his assistance.

[2] We take pleasure here to express our thanks to Prof. Ege of the Biochemical Institute for the privilege of using this fine polariscope.

rotation. As a precaution a zero reading with distilled water was taken each time.

Plasma-glucose was determined by the technique of Hagedorn and Jensen [Hagedorn, 1921][1]. When the concentration was high, the same amount of undiluted plasma was always used. After precipitation of the protein, the filtrate was divided into three parts and the sugar determined in each part separately, so that there was no danger of being out of range of the reagents. This procedure was found to be more accurate than diluting the plasma.

As an example of an experiment the following may serve.

The first injection consisting of 50 g. glucose and 0·8 g. creatinine in 500 cc. of water was ended at 10 h. 13. Blood was drawn at 10 h. 20, 10 h. 28½ and 10 h. 37. Urine samples were collected for the following periods: (1) 10 h. 17–10 h. 22, (2) 10 h. 22–10 h. 27, and (3) 10 h. 27–10 h. 39.

A second injection consisting of 50 g. glucose in 1000 cc. of water was ended at 11 h. 11.

Blood samples were drawn at 11 h. 22, 11 h. 35 and 11 h. 42. Urine samples were collected at (4) 11 h. 16–11 h. 20, (5) 11 h. 20–11 h. 25, (6) 11 h. 25–11 h. 30, (7) 11 h. 30–11 h. 35, and (8) 11 h. 37–11 h. 45.

The results of the analyses are given in the tables.

Urine samples	Amount (cc.)	cc. per min.	Creatinine (mg. per 100 cc.)	Glucose (mg. per 100 cc.)	Creatinine (mg. per min.)	Glucose (mg. per min.)
1	37·0	7·4	94·0	2500	6·96	186·0
2	10·0	2·0	236·0	3890	4·72	78·0
3	11·0	0·92	340·0	826	3·13	7·6
4	48·0	12·0	30·0	1420	3·60	170·0
5	38·0	7·6	35·8	1000	2·72	76·0
6	38·0	7·6	35·6	144	2·70	10·8
7	32·5	6·5	32·3	30	2·10	2·0
8	6·0	0·75	Lost	0	—	0

Blood samples	Creatinine (mg. per 100 cc.)	Glucose (mg. per 100 cc.)
I	5·0	592
II	3·6	324
III	2·7	225
IV	2·3	447
V	2·0	267
VI	Lost	156

The values of blood-creatinine and -glucose were then plotted in curves and from these curves the mean values for the various urine periods were read off. These values are used in the calculations, an example of which is given.

For the urine period (1) the excretion of creatinine was 6·96 mg. per min. For the same period the blood-creatinine averaged 5·08, so that in all 137 cc. of blood were cleared of creatinine per min. This amount is, according to the filtration theory, equal to the amount of filtrate formed in the glomeruli. For the same period the mean blood-sugar was 608 mg. per 100 cc., which means that 137 cc. filtrate contained 832 mg. glucose. 187 mg. were excreted

[1] We take pleasure to record our best thanks to Miss W. W. Smith for the determination of glucose in plasma.

and 645 mg. had thus been reabsorbed together with an amount of fluid which was 137 − 7·4 (the urine voided per min.) = 129·6 cc. The content of glucose in the reabsorbed fluid was $\frac{645 \times 100}{129{\cdot}6} = 498$ mg. per 100 cc.

Calculations like this lead to the following table:

For urine period	Average creatinine (mg. per 100 cc.)	Average glucose (mg. per 100 cc.)	Filtration (cc. per min.)	Filtered sugar (mg. per min.)	Re-absorbed sugar (mg. per min.)	Re-absorbed fluid (cc. per min.)	mg. per 100 cc. of glucose in re-absorbed fluid
1	5·08	608	137·0	832	646·0	129·6	498
2	4·26	450	111·0	498	420·0	109·0	385
3	3·12	172	100·0	172	164·4	98·2	168
4	2·39	502	151·0	757	587·0	139·0	422
5	2·29	440	119·0	523	447·0	111·4	322
6	2·17	371	124·5	461	450·0	116·9	386
7	2·06	302	102·0	308	306·0	101·3	302
8	1·86	172	—	—	—	—	—

$$\text{Filtration} = \frac{\text{mg. creatinine per min.}}{\text{mg. creatinine per 100 cc. blood}} \times 100.$$

$$\text{Filtered sugar} = \frac{\text{filtration} \times \text{blood-sugar}}{100}.$$

Reabsorbed sugar = filtered sugar − urine-sugar.

$$\text{Percentage of glucose in reabsorbed fluid} = \frac{\text{reabsorbed sugar}}{\text{reabsorbed fluid}} \times 100.$$

The results of a series of experiments of this kind are plotted in the figures and the results are the following.

(1) No difference can be shown between sugar excretion after one sugar injection and a second following shortly after the first. As seen in Fig. 1 the

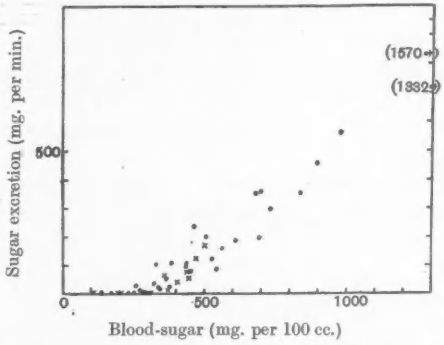

Fig. 1. Sugar excretion at various blood-sugar levels.
• = First injections. × = Second injections.

crosses representing sugar excretions observed after the second injection fall well in the middle of all the other points representing excretion after the first (or after a single) injection. Calculations of the amount of sugar which was reabsorbed at the various blood-sugar levels after the first and second injection

also failed to show any constant difference and we therefore must admit that the experiments do not show anything which can be explained as fatigue of either a secreting or a reabsorbing mechanism.

(2) The content of glucose in the reabsorbed fluid varies much. In the example given it varies between 168 mg. and 498 mg. per 100 cc., but even greater variations have been observed.

(3) The reabsorption of glucose goes on even when the blood-sugar is far above the normal. No regulation exists which stops the reabsorption.

(4) The reabsorption is not decreased with increasing blood-sugar.

(5) Sugar reabsorption thus goes on all the time, but the increase is less and less as the blood-sugar gets higher.

The higher the amount of filtered sugar, the smaller is the percentage of it which is reabsorbed. Up to an amount of 200 mg. per min. practically all

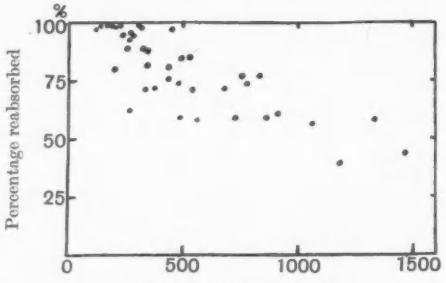

Fig. 2. The percentage of filtered sugar which is reabsorbed at various filtration rates.

is reabsorbed, but when about 500 mg. is filtered per min. only 75 % is reabsorbed and at a filtration of 1500 mg. per min. the reabsorbed amount is less than 50 % of the filtered.

We can next raise the question: what is the factor which limits the reabsorption?

Several factors perhaps work together. Possibly the amount of sugar which the tubule cells can transport per min. is limited, but it might also be possible that a maximum concentration difference between blood and urine could act as a limiting factor.

At a first glance it will perhaps seem contradictory to say so, as the concentration difference existing between urine and blood is such that the reabsorption ought to be favoured, because the concentration fall is from urine to blood. But if one tries to imagine how the reabsorption of sugar most likely is brought about, the possibility exists.

Experiments by White and Schmidt [1925] seem to indicate that the sugar is reabsorbed very soon after leaving the glomerular capsule. It is therefore conceivable and even probable that sugar is reabsorbed here until the concentration difference between blood and urine becomes too high. Some sugar

is left in the filtrate and this is then concentrated by reabsorption of water. The difficulty in trying to demonstrate this is that of course some water is reabsorbed together with the sugar. But as it is impossible to obtain any idea of how much this is, the only thing we can do is to calculate the percentage of sugar which would have been in the filtrate after reabsorption of the sugar if no water had been reabsorbed. This is done by the following formula:

$$\frac{\text{mg. urine-sugar per min.}}{\text{cc. filtrate per min.}} \times 100.$$

Example. In the experiment quoted the filtration is for the first period 137 cc. per min., and 832 mg. sugar are, according to the calculations, filtered and 645 mg. are reabsorbed, while 187 mg. are excreted. If we calculate the percentage of sugar in the filtrate after the reabsorption of sugar has taken place and assume that no water was reabsorbed we find $\frac{187}{137} \times 100 = 136$ mg. per 100 cc. In the same period the mean blood-sugar was 608 mg. per 100 cc.

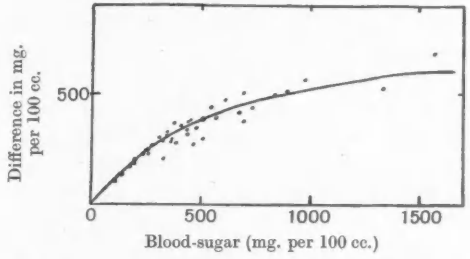

Fig. 3. The difference (calculated) in sugar concentration between blood and urine after reabsorption of sugar.

The concentration difference between blood and filtrate on this assumption was therefore $608 - 136 = 472$ mg. per 100 cc.

The results of calculations like this are shown in Fig. 3, which may perhaps be explained in the following way. As the blood-sugar increases, the reabsorption increases at the same time, but the tubule cells are not able to reabsorb sugar against a concentration difference higher than 600–700 mg. sugar per 100 cc.

It thus seems possible that one of the factors determining the sugar reabsorption may be the concentration difference between blood and urine at the place of reabsorption.

Summary.

1. The mechanism of glycosuria due to an increased blood-sugar level is discussed.

2. The results of a series of experiments bearing on this question are given graphically.

3. The experiments show that, according to the filtration-reabsorption

theory, the mechanism of glycosuria must be the failure of the reabsorption to increase as fast as the filtration of sugar increases with rising blood-sugar.

4. The percentage of sugar in the reabsorbed fluid varies within wide limits.

5. As a factor limiting the reabsorption of sugar we have probably the concentration difference between urine and blood at the site of reabsorption.

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CXVIII. NOTE ON MONTIGNIE'S ISOMER OF CHOLESTEROL, AND THE EFFECT OF HEAT ON CHOLESTEROL.

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Recently Montignie [1929] published a description of a new isomer of cholesterol, m.p. 137–137·5° and $[\alpha]_D$ − 19°. This isomer he obtained, along with cholesteryl cinnamate, m.p. 157–158°, and some unchanged cholesterol and cinnamic acid, by heating a mixture of two molecules of cinnamic acid and one molecule of cholesterol for ½ hour at 200°. He regarded the substance as a new isomer on the ground that, on treatment with alcoholic potash, it only suffered a very small loss in weight, actually, if his figures be correct, 4·18 %, and that it yielded an acetate, m.p. 103°, a benzoate, m.p. 131–132°, and a dibromide, m.p. 76°. He also stated that the substance gave the Liebermann reaction a little more slowly than ordinary cholesterol.

Wishing to examine this isomer more closely in the hope that it might prove useful in some other investigations on which we are engaged, we repeated Montignie's work, but though we obtained similar results, we were unable to confirm his conclusion that he had in hand a new isomer of cholesterol.

Experimental.

The cholesterol used was obtained from gall-stones and was purified through the dibromide in the usual manner. It melted at 148·8° and had $[\alpha]_D = -$ 39·28° in chloroform solution. It gave no reaction with Rosenheim's reagent, even after long standing.

10 g. of cholesterol were mixed with 8·08 g. of cinnamic acid and heated in a wide test-tube in a glycerol bath at 200° for ½ hour. The product was poured into hot alcohol. The difficultly soluble portion, after cooling to room temperature, was filtered off (fraction A). The filtrate, on cooling in a freezing mixture of ice and salt, deposited a rich crop of white crystalline matter, which melted unsharply at 137–138° (fraction B). This was the new isomer described by Montignie. A number of preparations were made, and the fractions B all had approximately the same unsharp melting-point, but the yield varied apparently with the volume of alcohol used.

Fraction A was boiled with ethyl alcohol, and the undissolved portion, m.p. 157–157·5°, consisted of pure cholesteryl cinnamate. The alcoholic filtrate

on concentration yielded crops of crystals, melting respectively at 150–151°, 146–146·5°, 145° and 140°.

Fraction B. Various preparations on precipitation from alcoholic solution by digitonin gave the following results:

	Substance (g.)	Digitonin compound (g.)	% sterol
(1)	0·024	0·0980	99·24
	0·0236	0·0964	99·30
(2)	0·0198	0·0756	92·82
(3)	0·0334	0·1206	87·78
(4)	0·0236	0·0932	96·00

The ether washings of the digitonin compounds on evaporation gave a solid residue. This residue was boiled out with water and filtered. The filtrate was faintly acid and contained a small quantity of cinnamic acid. The insoluble portion was recrystallised from ethyl alcohol. It melted at 156°, and was cholesteryl cinnamate.

Liebermann's reaction. The reaction was carried out under rigidly the same conditions with chloroform solutions of specimen (1) and of pure cholesterol of the same concentration. We could detect no difference by eye either in the colours produced in the two cases, or in the rate at which they developed.

Acetyl derivative. The acetate was prepared in the usual manner by boiling with acetic anhydride. After recrystallisation from alcohol it melted at 113–114°. On mixing with pure cholesteryl acetate there was no lowering of melting-point.

Benzoyl derivative. The benzoate was prepared by treatment of fraction B in pyridine solution with benzoyl chloride. After recrystallisation from alcohol it melted at 145–146° to a turbid fluid which became clear at 172°. On cooling it showed the colour changes characteristic of cholesteryl benzoate. Mixed with pure cholesteryl benzoate it showed no lowering of melting-point.

Dibromide. A portion of fraction B on treatment by Windaus's method in ethereal solution with a solution of bromine in glacial acetic acid gave a crystallised dibromide, which after washing and drying melted at 115°. On the other hand the same sample of B, after recrystallisation from alcohol, gave a dibromide, M.P. 123°, which decomposed at 125–126°. This on mixing with a specimen of pure cholesterol dibromide gave no lowering of melting-point.

On recrystallising fraction B from either methyl or ethyl alcohol, pure cholesterol was readily obtained.

It was also found possible by mixing pure cholesterol with small quantities of cinnamic acid and cholesteryl cinnamate, dissolving in alcohol, and freezing out the concentrated solution, to obtain a substance having all the properties of fraction B.

The mother-liquors obtained in the preparation of fractions A and B were evaporated to dryness. The dry residue was boiled out with water repeatedly and from the aqueous solution unchanged cinnamic acid, M.P. 133°, was obtained. The insoluble portion was easily separated by fractional crystallisa-

tion from alcohol into cholesteryl cinnamate and cholesterol. From these experiments we think it is clear that Montignie's substance was not an isomer of cholesterol, but an impure cholesterol, containing cinnamic acid and cholesteryl cinnamate.

On treatment, in chloroform solution, with Rosenheim's reagent (a saturated aqueous solution of trichloroacetic acid), none of the specimens of cholesterol, pure or impure, obtained in the above experiments gave any immediate coloration, but some of the specimens on long standing developed a pink colour. This was not due to any trace of allocholesterol, since on examining the solution with the spectroscope none of the bands of allocholesterol were observed. There was merely a slight general absorption. A specimen of allocholesterol, M.P. 117° and $[\alpha]_D + 4{\cdot}14°$, gave an absorption spectrum with a deep band at 4980 Å., and fainter bands at 5810, 5610 and 5330. On dilution the faint bands vanished, but the characteristic band 4980 persisted at a dilution fainter in colour than that given by the cholesterol specimens. In order to ascertain whether this pink colour may have been due to the effect of heat on cholesterol, a pure specimen of cholesterol was distilled in a high vacuum, others were heated at 185° *in vacuo* for many hours. The products after recrystallisation from alcohol melted at 148–148·5° and gave no colour with Rosenheim's reagent even on prolonged standing.

Specimens heated in open test-tubes at various temperatures from 160 to 200°, and for various lengths of time, after recrystallisation from alcohol melted at 148°, but with Rosenheim's reagent developed more or less rapidly a copper sulphate-blue colour, which was very persistent. The absorption spectrum was characterised by a well-marked band about 6600, and in some cases a fainter band about 6068. The results are given in the following table. The specimens of cholesterol used were obtained from either gall-stones or cod-liver oil, and were purified by the dibromide method and repeated recrystallisation from alcohol.

Source of cholesterol	Temp. of heating	Duration of heating	M.P. after recryst. from alcohol	Concentration of solution	Bands (middle)	
Cod-liver oil	160°	1 hour	148°	0·05 g. 5 cc. $CHCl_3$ 2 cc. trichloroacetic acid	6600	6076 (faint)
	200°	1 „	148°	0·05 g. 5 cc. $CHCl_3$ 2 cc. trichloroacetic acid	6682	—
	300°	½ „	148°	Not measured		
Gall-stones	180–185°	2 hours	148°	0·02 g. 5 cc. $CHCl_3$ 3 cc. trichloroacetic acid	6607	6070 (faint)
	175°	20 „	148°	0·1 g. 5 cc. $CHCl_3$ 3 cc. trichloroacetic acid	6600	6068 (faint)

Though the melting-point remained unchanged, the specific rotary power was depressed one or two degrees.

When cholesterol was heated at about 180° in a slow current of oxygen for 20 hours or more, a more profound change took place. An appreciable amount of CO_2 was produced and small quantities of acid substances distilled over. On

cooling, the melted mass set to a glass which did not crystallise on long standing. Though it contained a large proportion of unchanged cholesterol, this was difficult to crystallise from ethyl or methyl alcohol. A specimen recrystallised until it melted at 148° gave the blue colour with Rosenheim's reagent and its characteristic bands.

This reaction is under investigation and we hope to give a detailed account later.

REFERENCE.

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CXIX. THE ACTION OF ARSENATE ON HEXOSEPHOSPHATASE.

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(*Received July 4th, 1930.*)

According to the view of Harden and Young [1910] the rate of fermentation of sugar by yeast preparations is dependent, after initial esterification of the free phosphate, upon the rate at which phosphate is liberated by decomposition of the hexosephosphates formed; this takes place by means of an enzyme, hexosephosphatase, yielding a fermentable sugar and phosphate. They showed [1911] that, where hexosediphosphate is the only source of carbohydrate, the rate both of fermentation and of liberation of phosphate is greatly increased in the presence of arsenate; the accelerating effect of arsenate on the fermentation of sugar solutions by these preparations was therefore ascribed to a direct influence on the hexosephosphatase.

Neuberg and Leibowitz [1927] showed that the rate of hydrolysis of hexosephosphate by yeast in the presence of chloroform and toluene was increased five times by the addition of arsenate; they claimed that no fermentation of sugar took place, but gave no proof that in these conditions hexosephosphate was not fermented. Meyerhof [1927] has put forward the theory that hexosephosphate may also be directly fermented without previous hydrolysis, which might involve a different explanation of the action of arsenate.

Raymond [1928] showed that a decrease in the rate of hydrolysis by zymin occurs after washing, more marked in presence of arsenate; he suggested that there was a residual hydrolysis by the washed preparation, increased by the addition of coenzyme, or alternatively that the coenzyme could not be wholly removed by washing and was necessary for any reaction to take place. His experiments are open to criticism on several grounds, notably that the only reliable index of the absence of coenzyme is failure to ferment hexosephosphate, not merely sugar as in his experiments; moreover, he did not show that the washed zymin would ferment on addition of coenzyme. It has been found in this laboratory that it is often difficult, especially with top yeasts, to obtain a potentially active zymin free from coenzyme. It was accordingly decided to investigate the matter further.

The hexosephosphatase of washed zymin.

Dried baker's (Dutch) yeast was washed four times with eight parts of water, and dried with acetone and ether (DY_1). The washed material was tested for its fermenting capacity in a microfermentation apparatus at 30°.

Flask (*a*) 0·2 g. DY_1 + 0·25 cc. 40 % fructose + 0·5 cc. *M*/10 K hexosediphosphate + 0·05 cc. 2 *M* K_2HPO_4 + 1·5 cc. H_2O; (*b*) similar to above, substituting 1 cc. coenzyme solution for 1 cc. H_2O.

The coenzyme used was a 10 % boiled extract of yeast freed from phosphate by precipitation with lead acetate at p_H 6·0. No fermentation was observed in flask (*a*), while in (*b*) 4·65 cc. CO_2 were evolved in 2 hours. This method of testing for coenzyme was used throughout.

The hydrolysing power of the preparation (DY_1) was tested on various substrates and under different conditions. Series of flasks containing a weighed amount of DY_1 and the requisite solutions were placed in a thermostat at 30°. From each series a flask was removed at intervals, the action stopped by the addition of trichloroacetic acid and the free phosphate estimated by the Briggs method. Table I shows typical results.

Table I. *Hydrolysis by washed dried yeast.*

0·2 g. DY_1 in 2·0 cc. total volume, incubated at 30°.

	Substrate	Conc. Na_2HAsO_4 molar	Time (hours)	mg. P liberated per cc.
(*a*)	2 cc. H_2O	0	2	0·07
(*b*)	1 cc. 0·1 *M* K hexosediphosphate + 1 cc. H_2O	0	2	1·39
(*c*)	" " "	0·001	2	1·25
(*d*)	" " "	0	2	1·16
(*e*)	" " "	0·005	2	0·91
(*f*)	1 cc. 0·1 *M* K hexosediphosphate + 0·02 cc. toluene	0	2	1·28
(*g*)	1 cc. K hexosemonophosphate (Robison) + 1 cc. H_2O	0	1½	1·32
(*h*)	" " "	0·001	1½	1·07
(*i*)	" " "	0·005	1½	1·11

Similar results to the above were also obtained with a washed preparation of bottom yeast (Scotch). It seems certain, therefore, that hydrolysis of hexosemono- and di-phosphate can take place in the absence of coenzyme.

The interesting fact emerges from these experiments that under these conditions the hydrolysis is not accelerated by the addition of arsenate and is, indeed, slightly inhibited by its presence in concentrations which accelerate the fermentation of unwashed zymin. This indicates that the part played by the coenzyme, or by some other factor removed by washing, is intimately connected with the accelerating action of arsenate and not with the normal hydrolysis.

Starting from the premiss that the decomposition of hexosephosphate takes place only by a hydrolytic enzyme, it was obviously desirable to test the effect of coenzyme on preparations containing hexosephosphatase but no zymase, in order that the hydrolysis should not be masked by re-esterification. 100 g. of dried baker's yeast were incubated at 26° with 250 cc. water and 5 cc. toluene for 18 hours. After filtration on a Büchner funnel, the clear brown filtrate was evaporated to dryness in a vacuum desiccator; 120 cc. liquid yielded 14 g. of brown powder, readily soluble in water (DH_1). The dry preparation kept without change for several weeks; fresh solutions were made for each experiment.

A measured quantity of the enzyme was placed in each of a series of flasks together with the desired substrates and made up with water to a definite volume. The p_H was adjusted to 6·8 after saturation with CO_2. 1 cc. samples from each flask were transferred by an Ostwald pipette into a series of test-tubes placed in a thermostat at 30°. Tubes from each series were removed at intervals, the enzyme action stopped by the addition of trichloroacetic acid, and the contents transferred quantitatively to a 20 cc. flask and filtered. The inorganic P was then measured by the Briggs method. Identical samples were placed in microfermentation flasks and the CO_2 evolved was measured. A typical case is Exp. 29.

Exp. 29. The following solutions were used.
Enzyme. 10 % solution of dried autolysed yeast. DH_1.
Yeast extract. 10 % boiled extract of zymin.
Arsenate. 0·1 *M* Na_2HAsO_4.
Hexosephosphate. 0·1 *M* potassium hexosediphosphate.

	Substrate	mg. P liberated per cc.	cc. CO_2 per cc.
(*a*)	1 cc. enzyme + 2 cc. hex. phosphate + 3 cc. H_2O	0·17	0
(*b*)	" " " + 1 cc. yeast ext.	0·16	0·06
(*c*)	" " " + 0·3 cc. arsenate	0·10	0·01
(*d*)	" " " + 1 cc. yeast ext. + 0·3 cc. arsenate	1·19	0·52

This experiment was repeated many times with different preparations of the enzyme, giving substantially the same results, although the extent of the acceleration varied somewhat. The preparation therefore contained a hexosephosphatase but no coenzyme; with added coenzyme the hydrolysis was unaffected and a slight fermentation took place, though frequently there was a lag of 2–3 hours in CO_2 production. This lag proved misleading, as the earlier experiments were made over a period of only 1–2 hours and it appeared as if there were no fermentation, but it can only be concluded that in all cases there was a small amount of "zymase" present. The addition of arsenate in a concentration of 0·005 *M* caused a slight inhibition of hydrolysis, but by addition of both arsenate and coenzyme a striking acceleration of the rate of hydrolysis was produced, up to five-fold, together with an increased evolution of CO_2. The excess volume of CO_2 was equivalent to the excess of P liberated in the presence of arsenate over that in the normal hydrolysis, as shown in the following experiment:

Exp. 35. (*a*) 40 cc. 0·1 *M* K hexosediphosphate + 10 cc. enzyme + 10 cc. yeast ext. + 3 cc. H_2O.
(*b*) 40 cc. 0·1 *M* K hexosediphosphate + 10 cc. enzyme + 10 cc. yeast ext. + 3 cc. arsenate.
60 cc. of each were incubated at 30° for 4 hours.

	cc. CO_2	mg. P liberated
(*a*)	7·29	44·2
(*b*)	64·35	121·6
Excess (*b* − *a*)	57·06	77·4
Equiv. to 79·0 mg. P		

Purification of enzyme.

Various attempts were made to fractionate the preparation DH_1, and obtain a hexosephosphatase capable of acceleration with arsenate, but not of fermentation; none of these was successful. The enzyme was incubated for 16 hours at 26° at p_H 6·8; after this treatment it was found that the hydrolysis was only slightly decreased, while both the fermentation and the power to accelerate in the presence of arsenate had disappeared.

Exp. 31. 2 cc. 0·1 *M* K hexosediphosphate in total volume of 6 cc. Incubated 1 hour at 30°.

Substrate	mg. P liberated per cc.	cc. CO_2 per cc.
(*a*) 1 cc. enzyme + 1 cc. boiled yeast ext.	0·159	0·07
(*b*) 1 cc. enzyme + 1 cc. boiled yeast ext. + 0·3 cc. 0·1 *M* arsenate	1·052	0·52
(*c*) 1 cc. incubated enzyme + 1 cc. boiled yeast ext.	0·121	—
(*d*) 1 cc. incubated enzyme + 1 cc. boiled yeast ext. + 0·3 cc. 0·1 *M* arsenate	0·100	—

Alcohol precipitation. 70 cc. of crude hexosephosphatase solution, prepared from dried baker's yeast by autolysis, were treated with an equal volume of absolute alcohol. The white curdy precipitate was filtered on a Büchner funnel, washed with alcohol and dried in a vacuum desiccator over H_2SO_4, yielding 3·8 g. solid (*DHa*). 2 g. of this precipitate were ground with 40 cc. of water, dissolving almost completely, 1 cc. being equivalent to 0·92 cc. of the original extract. The hydrolysing capacity was then tested.

Exp. 65. 5 cc. 0·1 *M* K hexosediphosphate + 5 cc. enzyme (*DHa*) in total volume 15 cc.

Additions	mg. P liberated by 1 cc. enzyme in 80 mins. at 30°
(*a*) 5 cc. H_2O	0·546
(*b*) 0·5 cc. 0·1 *M* arsenate + 4·5 cc. H_2O	0·087
(*c*) 3·0 cc. coenzyme + 2 cc. H_2O	0·570
(*d*) 3·0 cc. coenzyme + 0·5 cc. 0·1 *M* arsenate + 1·5 cc. H_2O	0·066

0·92 cc. of the original extract liberated 1·09 mg. P in 80 minutes, so that only 50 % of the activity was retained in the precipitate. The inhibition of the enzyme by arsenate is in striking contrast to the acceleration obtained in the crude extract; addition of the alcoholic filtrate, after removal of the alcohol *in vacuo* at 30°, did not restore the power to respond to arsenate.

Purification by dialysis and by ultrafiltration in a Bechhold filter through collodion membranes had the same effect as alcohol precipitation; the residues, though retaining the same order of activity as the original and thus confirming the view that a dialysable coenzyme is not necessary for hydrolysis, were inhibited by arsenate even in the presence of added coenzyme and of the dialysate or ultrafiltrate.

The effect of fluoride.

Lipmann [1928], investigating the effect of fluoride on muscle extract prepared by incubation at 37°, stated that it inhibited the hydrolysis of hexosephosphate only slightly less than the lactic acid production from the

ester. It has been found here that the esterification of sugar by yeast preparations is inhibited by much smaller concentrations of fluoride than are required to affect the hydrolysis of hexosephosphate. The action of fluoride on the hexosephosphatase described above is very interesting, as the following experiment shows:

Exp. 33. 2 cc. 0·1 *M* K hexosediphosphate + 1 cc. enzyme (DH_1) in total volume of 6 cc. Incubated at 30° for 2 hours.

	Control		0·005 *M* NaF		0·01 *M* NaF		
Additions	mg. P liberated per cc.	CO_2 cc.	mg. P liberated per cc.	CO_2 cc.	mg. P liberated per cc.	% inhibition	CO_2 cc.
1 cc. yeast extract ...	0·336	0·0	0·344	0·0	0·225	24·5	0·0
1 cc. yeast extract + 0·3 cc. 0·1 *M* arsenate	1·502	0·51	0·853	0·25	0·440	70·5	0·1
mg. P excess in presence of arsenate	1·166	—	0·599	—	0·185	84·5	—

While a concentration of 0·01 *M* NaF causes a 24 % inhibition of the normal hydrolysis, 84 % of the increased liberation of phosphate due to the presence of arsenate is inhibited, together with approximately the same proportion of the CO_2 evolved.

Phosphatases from other sources.

Takadiastase contains an enzyme which hydrolyses hexosediphosphate very readily. It was found that this enzyme could not be accelerated under any condition by arsenate.

Exp. 34. 2 g. takadiastase suspended in 5 cc. H_2O.
1·0 cc. 0·1 *M* K hexosediphosphate + 1 cc. takadiastase, in total volume 3 cc.
Incubated for 3 hours at 30° at p_H 7·0.

Additions	mg. P liberated per cc.
(*a*) 0·5 cc. boiled yeast ext.	0·709
(*b*) „ „ + 0·3 cc. 0·01 *M* arsenate	0·180
(*c*) „ „ + 0·1 cc. „	0·306
(*d*) „ „ + 0·03 cc. „	0·406

A specimen of bone phosphatase was also examined and behaved in the same way as takadiastase.

Fractionation of coenzyme solution.

In the earlier experiments the coenzyme solutions used, 10 % boiled extract of zymin, were purified by precipitation with lead acetate at p_H 6·0, in order to remove the free phosphate which somewhat inhibits the hydrolysis. Later it was found that this process affected the accelerating mechanism to some degree, and the matter was therefore investigated more closely.

A 10 % boiled extract of zymin was treated with lead acetate at p_H 6·0 until no further precipitate was formed. The excess lead was removed with H_2SO_4 and the p_H adjusted to the original. 1 cc. of the filtrate = 0·5 cc. original. The lead precipitate was decomposed with H_2S and aerated, 1 cc. = 1 cc. original extract. The effect of the two fractions on the accelerating mechanism was then tested.

Exp. 29. 2 cc. 0·1 *M* K hexosediphosphate + 1 cc. enzyme (DH_1) in total volume 6 cc. Incubated for 1 hour at 30°.

	Additions	mg. P per cc. hydrolysed	mg. excess P over control
(*a*)	3 cc. H_2O	0·17	—
(*b*)	0·3 cc. 0·1 *M* arsenate + 2·7 cc. H_2O	0·114	− 0·06
(*c*)	0·5 cc. yeast ext. + 2·5 cc. H_2O	0·19	+ 0·02
(*d*)	,, + 0·3 cc. 0·1 *M* arsenate ...	0·38	+ 0·21
(*e*)	1 cc. Pb filtrate	0·14	—
(*f*)	,, + 0·3 cc. 0·1 *M* arsenate ...	0·26	+ 0·12
(*g*)	0·5 cc. Pb precip.	0·17	—
(*h*)	,, + 0·3 cc. 0·1 *M* arsenate ...	0·11	− 0·06
(*i*)	,, + 1 cc. Pb filt. + 0·3 cc. 0·1 *M* arsenate	0·39	+ 0·22

Both fractions were tested for coenzyme which was found only in the lead acetate filtrate. Some acceleration was produced on the addition of this fraction in the presence of arsenate, while the lead precipitate did not relieve the inhibition produced by arsenate. On adding both fractions together with arsenate, the acceleration was of the same order as that found with an equivalent quantity of the original extract. This points to some other factor besides coenzyme being concerned in the mechanism, and further experiments on these lines will be carried out.

All the results quoted above indicate that the action of arsenate in accelerating the rate of production of free phosphate from hexosediphosphate is not due, as was previously thought, to a direct effect upon the hydrolysis, but is part of a more complex system. The facts that the accelerated production of phosphate from hexosediphosphate was never obtained except with a parallel evolution of CO_2, and that the acceleration and the fermentation disappeared together on further treatment of the enzyme, suggest very strongly that the accelerating power of arsenate is related to the fermentation of hexosediphosphate rather than to its hydrolysis. A direct fermentation of hexosediphosphate was suggested by Meyerhof [1930] as an explanation of the fermentation of this substance by muscle and maceration extracts incapable of esterifying sugar. The possibility that the sugar formed by hydrolysis of hexosediphosphate is a labile form more readily fermentable cannot be completely ignored in this connection.

If the decomposition of hexosediphosphate takes place always by a hydrolysis to sugar and phosphate, it might be expected that fluoride would prevent the subsequent esterification, and so the fermentation of the sugar, without greatly affecting the rate of the preliminary hydrolysis; in presence of both arsenate and fluoride one might expect an increased liberation of phosphate and an accumulation of sugar. Actually, as shown, fluoride inhibits the increased phosphate production due to arsenate to the extent of 84 %, while the normal hydrolysis is only decreased 20 % by the same concentration of the salt.

While no definite conclusion that the accelerating action of arsenate on the liberation of phosphate is invariably accompanied by the fermentation of hexosediphosphate can yet be reached, the evidence here offered indicates

that the phenomenon is certainly more complex than has hitherto been realised.

I wish to record here my gratitude to Prof. Harden for his help and advice throughout this work.

SUMMARY.

1. The hydrolysis of hexosemono- and di-phosphate can be effected by washed dried yeast in the absence of coenzyme.

2. The accelerating action of arsenate on the rate of production of phosphate from hexosediphosphate takes place only in the presence of yeast extract and has not been obtained without an accompanying fermentation. Fluoride inhibits this acceleration.

3. Fractionation of the boiled yeast extracts used indicates that some other factor besides the coenzyme is necessary for the reaction.

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CXX. CHEMICAL CHANGE IN GELATIN RESULTING FROM THE METHOD OF STORAGE.

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(*Received July 7th, 1930.*)

The occurrence of intramolecular change in proteins as the result of physical treatment has been exemplified in earlier researches on gelatin, wherein it was shown, *inter alia*, that the dissolving of gelatin in water at 37° leads to a continuous irreversible change into the soluble form [Schryver and Thimann, 1927]. That the mere keeping of gelatin in a moist state also appears to produce chemical change is the subject of this note.

Previous investigations [Knaggs and Schryver, 1924] have shown that gelatin, after having been freed from ash, may be freed from colloidal impurities by flocculation in an electric field. In using this method it was found that by flocculation of a 1·5 % hydrochloric acid solution of gelatin at p_H 3, the crude gelatin may be freed simultaneously from ash and from colloidal impurity. Only simple apparatus is required, the platinum poles used being enclosed in vessels separated from the gelatin solution by semiperm[illegible] collodion membranes (Necolloidine) and washed constantly with a stream of water. As soon as the bulk of the inorganic matter and acid has been remov[illegible] by the current, the gelatin is precipitated as a gel, free of ash and colloidal impurities but not of soluble gelatin (ash content, 0·001 %). The soluble gelatin can, if desired, be afterwards separated by a similar flocculation from neutral solution, when the insoluble gelatin only is precipitated. It is a point of interest that the gel is periodic in structure, consisting of alternate layers of clear gel and opaque flocculum.

The gel obtained as above consists therefore almost entirely of true insoluble gelatin and soluble gelatin. It can be either stored as gel, under toluene, or dried down in a current of cold air, and these two different methods of storage apparently lead to different products.

It was pointed out in a previous communication [Thimann, 1930] that the amount of nitrogen precipitated from a gelatin hydrolysate by phosphotungstic acid offered a sensitive indication of chemical differences, if, and only if, it was used over a considerable range of nitrogen concentration. A curve similar to that described therein, *i.e.* nitrogen-concentration plotted against

the percentage nitrogen precipitated by phosphotungstic acid, was therefore determined for gelatin prepared as described above, and stored as gel (curve A). In another exactly similar preparation, starting from the same crude material, the gelatin was dried down immediately on completion of the experiment, and the curve again determined (curve B). Though the difference between these two products is only one of the method of storage, it will be seen that the second yields considerably more diamino-acid precipitate.

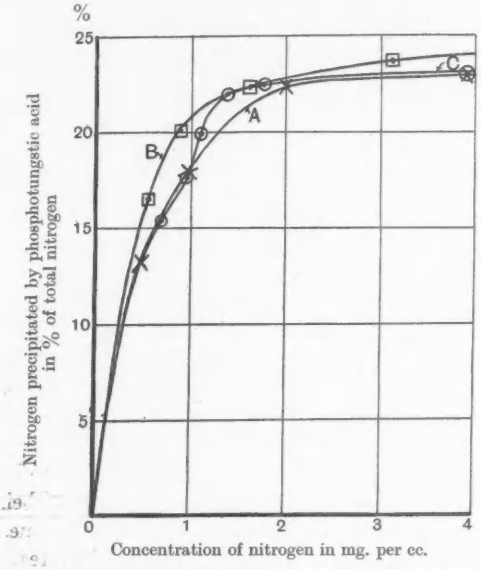

Fig. 1. Effect of storage of gelatin on diamino-nitrogen fraction.
× Stored as gel. ⊡ Stored dry.
⊙ Stored as gel and afterwards dried down.

In the hope of discovering whether the change is reversible, some of the gelatin stored as gel was afterwards dried down in a current of cold air. Recovery, apparently, did not take place, for the curve obtained (curve C) lies close to that for the gelatin preserved in the moist condition. Nevertheless, there is a curious irregularity, which may indicate some degree of reversibility in the change produced.

It appears, therefore, that moist storage produces a gelatin chemically different from the normal dry-stored product. Another explanation of the differences may lie in the fact that the dried material may be more slowly hydrolysed than the moist gel. If this were so, the curve for the dried material would correspond to that for gelatin exposed for a short time to acid treatment before hydrolysis, that is, it would lie, in general, above the normal curve, as indeed it does. If true this would indicate that for rigid comparison,

proteins must be in the same state of moisture or dryness before hydrolysis. Since, however, the hydrolysis was in all cases carried out by dropping the solid into the boiling acid, this suggestion does not seem probable.

Whichever of these explanations is the true one, the phenomenon further confirms, if confirmation be necessary, the extreme sensitiveness of the phosphotungstic acid precipitation over a range of concentration as an indicator of intramolecular change.

The variations in the curves are unfortunately too slight to allow of their being ascribed to any particular amino-acid.

A possible analogous difference between gelatin dried down as sol and as gel has been pointed out by Gortner and Hoffmann [1927] in regard to the degree of imbibition of water. This, however, although it may have a chemical cause, is a more purely physical phenomenon than the diamino-acid content, and may be explicable on a purely mechanical basis.

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CXXI. THE EFFECTS OF CYSTINE DIET ON KERATIN COMPOSITION IN RABBIT WOOL.

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(*Received June 26th, 1930.*)

The variability of the sulphur content of wool from type to type and along individual fibres may now be said to be firmly established [Barritt and King, 1926, 1929]. The factors influencing the sulphur content of a particular wool, *e.g.* breed, environment, diet, etc., have yet to be fully investigated, but the variation in sulphur content is obviously of special significance in the biosynthesis of wool keratin. The arguments in this connection put forward in 1927 [King, 1927] and in more detail in 1929 [Barritt and King, 1929] may be briefly summarised as follows.

In the coarser hairs of the fleece the three types of cell formation present, comprising scale, cortex and medulla cells, are all derived from the substantially sulphur-free plasmic material constituting the basal epidermal layer.

The finer fibres, which are composed only of scale and cortex cells, are higher in sulphur content than the coarser ones where the medullated core is present. A comparison of the sulphur content of non-medullated fibres with that of medullated, or kempy fibres of measured medulla content, affords indirect evidence that the medulla cells are substantially sulphur-free.

Thus it appears that the biochemical changes during keratinisation are accompanied by incorporation of sulphur in the case of the scale and cortex cells, but not in that of the medulla cells. This is in agreement with the histological view that the medulla channel is an upward protrusion of the basal epidermal layer. The medulla being sometimes continuous and sometimes intermittent indicates a continuous or intermittent requisitioning of sulphur-free material to make up for a deficiency in production of normal sulphur-containing cortex cells.

The variable "sulphur stimulus" must therefore be a highly important factor in hair growth, and the view that this is in the form of a cystine nucleus is supported by Rimington's proof [1929] that the sulphur present in wool and other animal hairs is wholly accounted for as cystine. In non-kempy wools the extremes of sulphur content so far observed are 2·93 % and 4·11 %, calculated on the dry weight, corresponding to 10·98 % and 15·41 % of cystine respec-

tively. The relative quantities of cystine and glutamic acid found in wool hydrolysate are in fair agreement with the supposition that the actual keratinising agent is glutathione.

This variable incorporation of the cystine nucleus, apart from its influence on textile properties, will obviously enter into biological problems of wool production, and will be determined partly by hereditary and partly by environmental and pathological conditions. If, as Robertson and Marston [1928] claim, wool keratin is of constant sulphur content, any factor inducing in the fleece an increase in keratin-sulphur would be reflected merely in an increased weight of fleece.

If the variability recorded by Barritt and King [1926, 1929] is not entirely an hereditary effect suitable modifications in diet may be expected to modify not only the total fleece weight, but also the percentage of sulphur in the wool.

Regarding the effect of diet it has recently been recorded by several observers [Hartwell, 1921, 1925; Evvard, Fox and Guernsey, 1914] that the cystine content of the diet is a limiting factor in the growth of hair, *e.g.* rats fed on a low cystine diet suffer a loss of hair which can be restored by a normal diet. All these observations have, however, been general in character and information as to any variation in the sulphur content of the hair with variation in the cystine content of the diet is lacking.

The purpose of the present experiments was to observe the effect, if any, on the sulphur content of the fur of rabbits resulting from a varying cystine diet. Angora rabbits were selected because of the ease of control of the experiments and the quick growth of the fur.

Experimental.

The 24 rabbits employed were all white Angora bucks of adult age and similar strain. They were housed and fed in a manner which prevented matting and soiling of the wool.

The rabbits were fed on a normal diet consisting of whole oats in the morning, and in the evening of poor meadow hay, turnips and a damp mash of broad bran and flaked maize. Grass replaced the turnips and hay from the beginning of June to the conclusion of the experiment. The average amount of food consumed by each rabbit per day was as follows:

Whole oats	...	34 g.	Hay ...	...	54 g.
Broad bran	...	16	Turnips	...	96
Flaked maize	...	32			

The first group of six rabbits was maintained on this diet. With the second, third and fourth groups this was supplemented by 0·05, 0·1 and 0·2 g. of cystine respectively.

The cystine was obtained from the hydrolysis of wool as described by Barritt [1927], 400 g. being used for each hydrolysis. The cystine was not entirely freed from tyrosine and traces of inorganic matter and had a sulphur

content of 25 % to 26 % for the various samples prepared. (Cystine contains 26·67 % S.)

The requisite amounts of cystine were thoroughly mixed into part of the bran ration and fed to the rabbits, the rest of the diet being offered as soon as this was eaten. This method ensured the eating by each rabbit of the full dose of cystine each day.

The rabbits were clipped on April 18th (the date of clipping prior to April 18th was January 3rd, 1929) and on July 11th, 1929 (a growth period of 84 days), the wool being removed as close to the skin as possible. The fleece was weighed to the nearest g. and 20 g. were taken at random from the whole fleece for the analysis.

The rabbits were weighed daily for 5 days at the beginning and conclusion of the experiment, the figures quoted being the average of these weights. Undue importance should not, however, be attached to these, as it has been found [Pickard, 1928] that the weight of the rabbit has little, if any, influence on the wool yield.

It is difficult to compare the weight of the April clip with that of the succeeding quarter, as the seasonal variation in the wool yield is considerable.

The animals remained in good health throughout the experiment. The wools were sampled, purified and analysed as described in earlier papers [Barritt and King, 1926, 1929].

The results are given in Table I.

Table I.

		Beginning of experiment				End of experiment		
Group	No. of rabbit	Weight of rabbit (kg.)	Weight of clip (g.)	Sulphur on dry weight %	Cystine per day (g.)	Weight of rabbit (kg.)	Weight of clip (g.)	Sulphur on dry weight %
I	103	2·094	47	5·10	(Controls)	1·924	35	5·05
	140	2·434	70	4·90		2·434	47	5·07
	237	3·057	96	4·86		3·453	62	5·06
	243	2·378	59	4·64		1·953	30	4·98
	301	2·038	33	4·29		2·717	35	5·03
	302	2·491	64	4·12		3·056	35	4·79
II	5	3·113	70	5·22	0·05	3·226	35	5·23
	22	3·170	56	4·99		3·198	54	4·98
	75	2·462	47	4·98		2·321	34	4·93
	226	2·378	55	4·66		2·604	31	5·16
	229	2·349	61	4·93		2·377	27	5·21
	254	2·434	77	4·44		2·773	51	4·97
III	28	2·745	49	—	0·1	2·660	35	—
	63	2·378	58	4·93		2·434	36	5·11
	214	2·858	58	—		3·170	45	—
	218	2·802	58	4·84		3·283	30	4·99
	224	2·349	76	—		2·887	54	—
	235	2·575	69	4·77		2·660	45	4·91
IV	107	3·170	75	—	0·2	3·283	57	—
	193	2·887	72	4·92		2·717	37	5·02
	221	2·378	84	—		2·321	52	—
	238	2·123	49	4·85		2·434	41	5·05
	242	2·292	47	—		2·773	30	—
	253	2·292	40	4·57		2·717	32	4·89

Discussion of results.

The results indicate very clearly a large seasonal variation in almost every sample, there being in the majority of cases an increase in the sulphur content; in the case of rabbit no. 301 the increase was almost 18 %. The limiting value for sulphur content in this type of rabbit (Angora) appears to be about 5·2 %.

The results thus support the previous work of Barritt and King [1926, 1929] on the variable sulphur content in wool fibre, particularly variation along its length, which may thus also be a seasonal effect. They further illustrate the variability in another type of hair keratin, as opposed to the finding of Robertson and Marston [1928] that the sulphur content of wool keratin is constant. A further point of interest, as to whether in the cases of increased sulphur content the sulphur is still wholly accounted for as cystine is at present under investigation.

In so far as the effect of the cystine itself is concerned no real differences in sulphur content are to be found between the control rabbits and those fed on the cystine-rich diet, and evidence that feeding of cystine as such has any effect on the sulphur values is at present lacking. It was observed, however, that among the twelve rabbits comprising the controls and those having 0·05 g. cystine per day, four developed suspension of fur growth on the back, whereas among those fed with 0·1 and 0·2 g. cystine per day, no such suspension occurred.

The negative results may be variously explained as due to:

(1) the cystine having been fed as cystine itself and not as protein-cystine;

(2) the amount of cystine fed being insufficient to give any appreciable effect;

(3) the period of the experiment coinciding with the maximum natural seasonal variation, thus masking the effect of the additional cystine.

It is proposed to continue the experiments by increasing the amount of cystine; also by giving diets rich in plant-cystine, and by measuring the effect over the 3 months' period which in the present series has been found to correspond with a naturally low sulphur content.

Summary.

1. Angora buck rabbits have been fed for 3 months on a normal diet and on diets with the addition of varying amounts of cystine obtained from wool hydrolysate.

2. In general, a large increase in the sulphur content of the fur of individual rabbits has been found between the two successive 3-month periods, January to April, and April to July, in the controls as well as in the cystine-fed animals. The maximum increase observed was 18 % and this occurred with rabbit no. 301 on normal diet throughout.

3. While the fleece weights are generally less for the shorter second period, the fleece weight ratios for the two periods are substantially the same with the cystine-fed animals as with the controls.

The authors' thanks are due to Mr J. A. F. Roberts, through whose introduction their collaboration was initiated.

Mr A. N. Davidson has ably assisted in the analytical work.

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CXXII. OXIDATION-REDUCTION STUDIES IN RELATION TO BACTERIAL GROWTH.

I. THE OXIDATION-REDUCTION POTENTIAL OF STERILE MEAT BROTH.

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(*Received June 30th, 1930.*)

Several observations have been made on the oxidation-reduction (O/R) potentials of growing cultures of bacteria, but the O/R potential associated with the initiation of bacterial growth has been studied only by Fildes [1929, 1] in connection with the germination of spores of *B. tetani*.

Developing the views of Clark [1924] Fildes pointed out that "the term 'anaerobe' as applied to a micro-organism implies that it requires for growth a certain reducing intensity in its surroundings." Conversely, for organisms which will not grow under "strictly anaerobic" conditions, it may be assumed that the reducing intensity must not be too great if growth is to take place.

The relation of the onset of bacterial growth to the oxidation-reduction conditions in the surroundings is thus a subject which has a direct bearing on the biology of bacteria and, further, has been used by Fildes [1929, 2] to account in part for their pathogenic action.

In studying the effect of p_H, the hydrogen ion concentration of a culture medium can be adjusted to any value and maintained constant against the CO_2 of the air, etc., by means of buffers. In the case of the O/R potential, which depends, at constant p_H, on the degree of reduction of some electromotively active system, anything which tends to alter the degree of reduction will alter the potential. The interference of fortuitous oxidising or reducing agents with the normal behaviour of the oxidation-reduction systems of culture media must therefore be strictly controlled. The O/R potential of a culture medium has no definite significance until such factors as the casual access of the oxygen of the air are eliminated. An essential preliminary to any attempted control of oxidation-reduction conditions is, therefore, an investigation of the O/R potential of sterile culture media. Until this is done it is not possible to control the level of O/R potential in a medium.

There are obvious limitations and difficulties in the investigation of such problems by the O/R dyes used by Fildes and others. It was therefore decided to use electrometric methods.

[1] Working under a grant from the Medical Research Council.

The ability of sterile meat broth and other bacteriological media to reduce various O/R dyes, notably methylene blue, has long been known. Only recently have attempts been made to measure this reducing intensity in terms of O/R potential. Dubos [1929] used Clark's oxidation-reduction dyes and found that plain broth under vaseline seal reduced potassium indigodisulphonate almost completely at p_H 7·8, corresponding to an E_h of about − 0·18 volt (E_h is the potential referred to that of the normal hydrogen electrode as zero). Fildes [1929, 1] made more extensive observations over a range from p_H 6 to 9 and showed that the p_H had an important effect not only on the rate of reduction but also on the final level of potential which the medium attained. His value at p_H 7·8 approximates to that of Dubos.

Coulter [1928] is the first to have applied electrometric methods to the study of the oxidation-reduction system of sterile broth. His experiments show clearly that there is an electromotively active system present in sterile broth which tends to establish an "inert" electrode at a definite level of E_h (at constant p_H) when access of oxygen is prevented. When a sample of broth was de-aerated with oxygen-free nitrogen the potentials of gold electrodes immersed in the broth steadily became more negative. The drift of potential was slower if the de-aeration took place only for a short time and the vessel was then sealed off. In either case Coulter found that the potential reached a final value of about E_h − 0·06 volt at p_H 7·6. This is about 0·1 volt more positive than the value found by Fildes and by Dubos, using dyes.

Unfortunately Coulter confined his observations to one p_H value. The present paper gives a more extended view of the electromotively active system of broth. The general behaviour of the system described by Coulter is fully confirmed. There is, however, a quantitative difference between his value of the potential at p_H 7·6 and the corresponding value found in the present work. This may be due to differences in the actual medium used.

Experimental.

The general technique for oxidation-reduction potential measurements described by Clark [1923], Clark and others [1928] has been used, with some alterations necessitated by the special nature of the system to be investigated. The electrode vessel consisted of a stout Pyrex vessel 4 cm. in diameter and 10·5 cm. long, having a capacity of about 100 cc. The vessel was closed by a large rubber bung carrying three electrodes, gas inlet and outlet tubes, and a saturated KCl-agar bridge for making contact with the standard half-cell. The half-cell used in these experiments was quinhydrone in $M/20$ phthalate. This was found to have a value of E_h + 0·445 volt at 38°, which was the temperature of the incubator enclosing the electrode vessel and half-cell.

The electrodes were plates of platinum foil, area 4 cm.^{2}, freshly gilded, electrolytically, for each experiment. The pieces of foil were welded to platinum wires and these fused into glass tubes, contact being made with mercury. Care must be taken to avoid minute cracks in the glass at the seal. These may

cause large discrepancies between electrode readings. The electrodes were cleaned in chromic-sulphuric acid before gilding. Care was taken not to finger the electrodes after they had been gilded and they were kept immersed in distilled water. Occasionally bright platinum electrodes were used. The bung carrying the electrodes, etc., was sterilised by autoclaving, supported loosely in a conical beaker with the electrodes under distilled water and the mouth of the beaker wrapped with cotton gauze. The electrode vessel, containing 50 cc. of buffer-broth, was autoclaved separately. The KCl-agar bridge carried by the bung was drawn off and sealed at both ends before autoclaving.

After autoclaving, the capillary ends of the agar bridge were cut off with a sterile glass-knife and the bung was fitted aseptically to the electrode vessel. The bung was painted with gold size when fitted, to make it gas-tight. The gas inlet tube contained a small plug of cotton wool to filter the gas. Oxygen-free nitrogen was used for stirring and de-aerating the electrode vessel and its contents. The nitrogen, from a cylinder of the compressed gas, was purified by passage through reduced copper gauze contained in a silica tube heated to a red-heat by an electric furnace. The rigorous precautions against leaks of oxygen recommended by Clark [Clark and others, 1928], including a copper tubing gas-line and elimination of rubber connections were adopted. A vigorous stream of nitrogen was used for about 20 minutes, and was then reduced to give a sufficient degree of stirring and de-aeration.

The potentials were measured by the valve potentiometer system described by Harris [1928]. This system has the advantage of avoiding polarisation of the electrodes when finding balance. This is a point of great importance in measuring the potentials of such a poorly poised system as that in broth.

Sterile buffered broth.

The broth used throughout these experiments was the standard laboratory medium: peptone-beef infusion (T. Morson and Sons' peptone). No glucose was added but native sugars were not fermented out. The broth was made of double strength for storage. Immediately after preparation, the batches of broth were tubed in 30 cc. lots, and while still hot from the autoclave placed for storage in anaerobic jars. For the experiments this broth was diluted with an equal volume of $M/10$ buffer [Clark, 1928] immediately before use. Several batches of broth were buffered with $M/10$ KH_2PO_4 + asparagine. With this the range p_H 6·5 to 9·5 could be conveniently covered by the one buffer mixture.

A hydrogen electrode titration was done on a sample of buffered broth from each batch. Samples of the broth (50 cc.) could then be made up to any required p_H by adding the required amount of standard alkali or acid read from the titration curve. The subsequent autoclaving altered the p_H a little. The exact p_H of each sample was, therefore, always determined by the hydrogen electrode after the drift experiment had been completed.

Character of the potentials.

Typical curves showing the change of potential with time when sterile broth is de-aerated are given in Fig. 1. When readings of the potential were

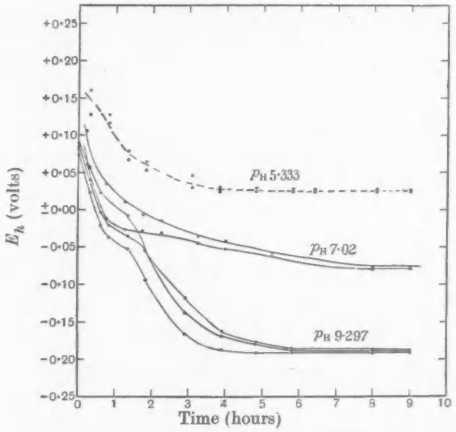

Fig. 1. Time-potential curves of sterile buffer-broth de-aerated with pure nitrogen.

made soon after the beginning of de-aeration, large differences between individual electrodes were observed. These tended to come together as the potential slowly became more negative. The electrodes never exhibited the precise agreement that is found with well-defined, well-poised systems. In general, with the electrodes prepared as described above, readings agreeing to within 10 mv. or less were finally obtained. Occasionally one electrode of the three was markedly different (this was generally found to be due to a cracked electrode); the closely agreeing values of the other two were then taken. The potentials of the three electrodes plotted against time gave individually smooth curves, tending to come together as the potential reached its limiting value after 6–8 hours.

The potentials of the system are extremely poorly poised, as shown by the ease of polarisation if a simple potentiometer circuit is used. With the valve potentiometer this was avoided. The potential to be measured was made to contribute to the negative grid bias of one of two balanced valves. The potential chain

− Au-plated Pt	Broth	KCl-agar	M/20 Phthalate, quinhydrone	Au-plated Pt +

was therefore effectively on open circuit during balancing. Even with this circuit, if the chain was left switched in, there occurred a very slow positive drift. The cause of this is unknown. It may be a further indication of the small

capacity and feeble poising of the electromotively active system in the broth. Possibly the charge on the electrode in the broth cannot quite be maintained by the small amount of active material available. An interesting side-light is thrown on this by the observation that this slow positive drift was much reduced if the area of the electrode was increased. Thus, while the drift was as much as 5 millivolts per minute with small pin electrodes of area 0·5–1 cm.², with plate electrodes of area 3–6 cm.² the drift was less than 1 millivolt in 3 minutes. This was negligible during the time necessary to switch in the chain and take a reading. As a routine, the cells were left on open circuit until it was necessary to take a reading.

Time-potential curves were observed in a large number of experiments with sterile buffered broth from p_H 4 to 10.

Relation of E_h *to* p_H.

If the final levels of potential to which the broth drifts (Table I) are plotted against the corresponding p_H values, an interesting relation is obtained (Fig. 2).

Table I. *Relation of* E_h *of completely de-aerated buffer-broth to* p_H. *Observations with electrodes in absence of O/R dyes.*

(E_h is the mean of three electrodes.)

p_H	E_h (volts)	Buffer	p_H	E_h (volts)	Buffer
4·25	+ 0·070	Phthalate	7·084	− 0·099	Phosphate
4·82	+ 0·048	,,	7·31	− 0·092	,,
5·23	+ 0·023	,,	7·35	− 0·100	,,
5·96	+ 0·005	Phosphate-asparagine	7·37	− 0·110	,,
5·99	± 0·000	Phosphate	7·57	− 0·130	,,
6·05	+ 0·010	,,	7·62	− 0·130	Phosphate-asparagine
6·06	+ 0·015	Phosphate-asparagine	8·26	− 0·165	Phosphate
6·42	− 0·025	,, ,,	8·27	− 0·167	,,
6·59	− 0·003	Phosphate	8·45	− 0·185	Phosphate-asparagine
6·63	− 0·030	,,	8·46	− 0·166	Borate
6·82	− 0·055	Phosphate-asparagine	8·57	− 0·181	Phosphate-asparagine
6·89	− 0·048	Phosphate	9·30	− 0·190	Borate
6·92	− 0·041	Phosphate-asparagine	9·68	− 0·225	,,
7·00	− 0·050	Phosphate	10·47	− 0·258	,,
7·02	− 0·083	,,			

This curve at once recalls that given by a reversible system at any fixed degree of reduction in its relation with p_H. It may be said at once that the points on this E_h–p_H curve do not align with the precision that a well-defined reversible system gives. This lack of precision was particularly marked, in a few cases, with broths which had not been stored anaerobically, although these generally gave points on the E_h–p_H curve (Table I, phosphate-asparagine buffered broth). The discrepancy was as much as 40 mv. in three experiments. This was within the range p_H 6·5–7·5 (*i.e.* on the steepest part of the E_h–p_H curve); and further, since added dye was not reduced so much as usual, slight leaks of oxygen were suspected in these cases. This would have its greatest effect over the steep part of the curve.

The smooth curve drawn through the points has been given standard slopes in order to compare it conveniently with analogous curves. It is

striking that such a good fit is obtained in this way. The three sections of the curve are not due to the three different buffers used, since points obtained with phosphate and with phosphate-asparagine buffers occur on all sections of the curve.

For the broth system it is a fair assumption that this curve represents the relation with p_H at a high degree of reduction. Attempts to obtain a more negative potential level by reducing the broth with hydrogen and platinised asbestos have not succeeded. The potential level obtained was the same, within experimental error.

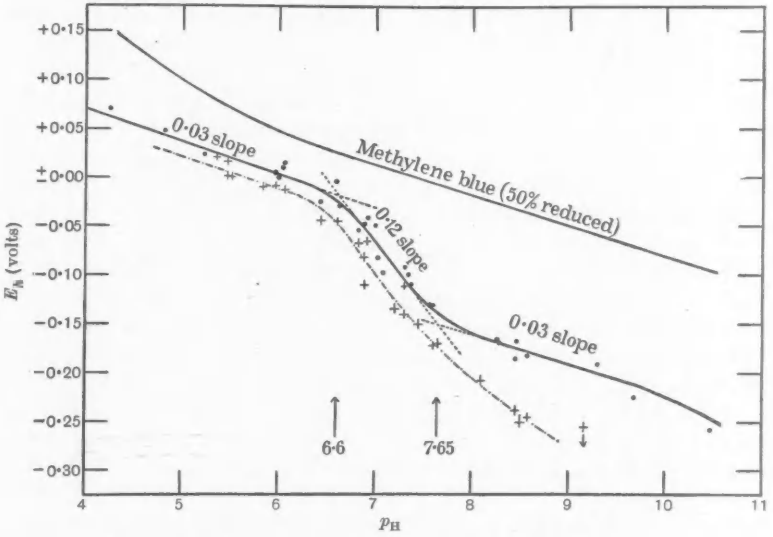

Fig. 2. Relation of E_h of completely de-aerated buffer-broth to p_H.
Full curve —•—•—•—•—: E_h by electrodes alone in broth.
Broken curve —+ - - - + - - - + - - -: E_h by dyes (colorimetrically and electrometrically).

Cannan, Cohen and Clark [1926] obtained a somewhat analogous curve by plotting certain potential levels of washed yeast suspensions against p_H.

If it be accepted that the curve of Fig. 2 does express a definite relation between p_H and some fixed degree of reduction of the electromotively active system of broth, the inflexions of the curve may be partially interpreted. By analogy with the behaviour of truly reversible systems [Clark and others, 1928], the inflexions indicate the dissociations of hydrions of the system. These dissociations may be approximately located at p_H 6·6 and 7·65.

The value of E_h at p_H 7·6 is −0·13 volt compared with Coulter's value of − 0·06 volt. It may be pointed out that the p_H at which Coulter was working falls within the zone in which I obtained most variation in the values for the final level of E_h.

The difference may be due to differences in composition of the media, or it is possible that the p_H of Coulter's medium was not exactly 7·6 after autoclaving. I have found that autoclaving even a buffered broth at slightly alkaline p_H tends to shift the p_H towards the acid side. The E_h–p_H curve shows that a slight change of p_H in the region 6·5–8 causes a considerable change of E_h, but the difference of E_h is almost too great to make the latter explanation fully satisfactory.

Correlation of E_h *determined by oxidation-reduction dyes and that determined electrometrically.*

The relative facility with which the oxidation-reduction dyes can be used has led to their adoption for measuring the reducing intensity of cells, tissues and culture media, but these measurements have seldom been correlated with electrode measurements on the same material in the absence of dyes. This has been done in the present case. Samples of the same batches of sterile buffered broth as used for the electrode measurements were de-aerated by the same technique in a duplicate apparatus. A quantity of dye to give a suitable colour for observation was added to the broth, and its degree of reduction determined colorimetrically. Colour standards were made by suitably diluting portions of this broth. In the case of dyes which had a noticeable colour when reduced in broth (*e.g.* the indigosulphonates) the colour of the partially reduced dye in broth was matched with the combined tint of two tubes, one of which gave the percentage oxidised tint and the other the complementary percentage reduced tint.

Arrangements were made whereby samples of the broth + dye, which was being de-aerated, could be drawn off from time to time, with exclusion of oxygen, and its colour matched with the standards. In this way, potential-time curves analogous to those observed electrometrically were obtained. At any p_H, if one dye became practically completely reduced, the next lower dye on the E_h scale was used as a check (Table II A). Ample time was allowed, so that the poising effect of the dye should not give an apparent final E_h level. The points thus obtained by dyes for the final level of E_h in de-aerated broth are plotted in Fig. 2. It is seen that the dye method gives, under controlled conditions of p_H and rigorous exclusion of oxygen, a curve sensibly parallel at first to the curve obtained by electrodes alone, but about 0·02 volt more negative. This holds, however, only over the range up to p_H 7·5. At more alkaline reactions than this the curves diverge, and a dye is reduced to an increasingly more negative E_h than would be indicated by an electrode alone in the same broth.

Electrodes in broth + dye indicate the dye potentials. Points obtained in this way are shown in Fig. 2 and afford a more precise measurement than the colorimetric method (Table II B).

A similar divergence between the indications of electrodes and dyes is seen, for example, with cysteine and other sulphydryl compounds. A cysteine

Table II. *Relation of* E_h *of completely de-aerated broth to* p_H. *Observations with O/R dyes.*

A. *Colorimetric observations.*

p_H	% reduction of dye (observed)	E_h (volts) (calculated)	Buffer
5·35	85 indigo 4*	+ 0·02	Phthalate
5·46	60 ,, 3	+ 0·001	,,
5·46	84 ,, 4	+ 0·016	,,
5·84	85 ,, 4	− 0·01	,,
6·60	80 ,, 4	− 0·045	Phosphate
6·87	65 ,, 3	− 0·082	,,
6·87	35 ,, 2	− 0·111	,,
7·2	50 ,, 2	− 0·134	,,
7·3	50 ,, 2	− 0·140	,,
7·45	60 ,, 2	− 0·150	,,
7·65	77 ,, 2	− 0·170	,,
8·09	85 ,, 2	− 0·207	,,
9·16	98 ,, 2	− 0·255	Borate

B. *Electrometric observations (electrodes + dyes).*

p_H	E_{h_1}. No dye (observed)	E_{h_2}. Dye present (observed)	$E_{h_2} - E_{h_1}$	% reduction of dye (calculated)	Buffer
5·96	+ 0·005	− 0·009	0·014	76 indigo 4*	Phosphate-asparagine
6·06	+ 0·015	− 0·013	0·028	77 ,, 4	,,
6·42	− 0·025	− 0·045	0·020	89 ,, 4	,,
6·82	− 0·055	− 0·067	0·012	76 ,, 3	,,
6·92	− 0·041	− 0·065	0·024	29 ,, 3	,,
7·31	− 0·092	− 0·111	0·019	9 ,, 2	Phosphate
7·62	− 0·130	− 0·171	0·041	82 ,, 2	Phosphate-asparagine
8·45	− 0·185	− 0·238	0·053	98·5 ,, 2	,,
8·57	− 0·181	− 0·245	0·064	99 ,, 2	,,

* Indigo 4 is indigotetrasulphonate.
,, 3 is indigotrisulphonate.
,, 2 is indigodisulphonate.

solution will reduce a dye to a value of E_h more negative than the cysteine solution alone would show. It is evident that much caution must be used in the interpretation of either electrode or dye measurements alone in any particular set of experiments.

In connection with this difference between the potential indicated by dyes and by electrodes, some experiments of Wurmser and Geloso [1928, 1929] are of interest. They investigated the potential of glucose solutions in buffers with electrodes, both in presence and absence of dyes. They found that the electrodes alone would eventually, after 5 or more days, reach approximately the same level of potential as was reached much more rapidly in the presence of dyes. This work has been only very briefly reported, without experimental details, and requires confirmation. In the present work, the potential had reached a definite level in 8–10 hours, after which time, in several prolonged experiments of 20–30 hours, no further change took place. From the regularity of the relation of E_h to p_H (Fig. 2) it is evident that the potential level attained in completely de-aerated broth after 8–10 hours is definite and significant and not purely arbitrary.

SUMMARY.

The object of the present investigation has been to establish some fundamental requirements of technique preliminary to the application of the ideas of oxidation-reduction potential theory to bacterial systems. To this end the behaviour of sterile meat broth has been studied.

The O/R potential of completely de-aerated, sterile, buffered meat broth has been measured over the range p_H 4–10·5.

The electrode potentials have been compared with those indicated by Clark's O/R dyes.

It is found that the two methods do not indicate the same potential level, a dye indicating a more negative potential than would be shown by an electrode in broth alone.

Quantitative data are provided that should assist the characterisation of the electromotively active system of broth.

I am deeply indebted to Dr P. Fildes for his interest in initiating this work. My best thanks are due to Prof. R. K. Cannan for much valuable advice and criticism.

The expenses of this investigation were defrayed by the Government Grants Committee of the Royal Society and the Yarrow Research Fund of the London Hospital.

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CXXIII. OXIDATION-REDUCTION STUDIES IN RELATION TO BACTERIAL GROWTH.

II. A METHOD OF POISING THE OXIDATION-REDUCTION POTENTIAL OF BACTERIOLOGICAL CULTURE MEDIA.

By BERT CYRIL JAMES GABRIEL KNIGHT[1].

From the Bacteriological Department, London Hospital.

(*Received June 30th, 1930.*)

In the foregoing paper [Knight, 1930] the oxidation-reduction (O/R) potential of sterile meat broth was studied. It was shown that the potential of the broth slowly drifted to a definite level, depending on p_H, when oxygen was removed. It is obvious, therefore, that if it is desired to study the behaviour of a micro-organism under constant conditions of O/R potential of its surroundings, this can only be done at the final level of potential to which a broth will drift, unless a method can be devised whereby the potential can be held constant at any level.

In seeking some method of poising the O/R potential of a medium at a chosen level several possibilities at once present themselves. Thus, the presence of an O/R dye tends to poise the potential temporarily in the region of 50 % reduction [Cannan, Cohen and Clark, 1926]. The broth soon completes the reduction of the dye however, and the negative potential drift of the medium proceeds as before until it reaches its final level. The time during which the dye poises the potential in the region of 50 % reduction can be prolonged by increasing the amount of dye, but there are obvious practical limits to this method. There is the further objection that the potential levels would be limited to the number of dyes and other O/R systems available. Also there might be a specific chemical effect of the dye on a particular micro-organism.

A second possibility would be to destroy or remove or completely oxidise the system responsible for the potential and then to poise at a chosen level by a suitable degree of reduction of an added O/R system. In the absence of oxygen and of any substance in the broth to react with the added system, the potential could then be held constant.

Attempts to oxidise completely the system by prolonged passage of oxygen have not been successful; neither have attempts to destroy or to remove it, without altering the nutrient properties of the medium.

[1] Working under a grant from the Medical Research Council.

A suitable synthetic medium to which a definite O/R system might be added is a further possibility, as yet unrealised.

It becomes apparent that static methods of poising in the presence of such a system as that in broth are at present unavailing, and that some dynamic method is necessary. That is, the negative drift of the medium must be counterbalanced by some opposing oxidising tendency. It is on these lines that the method to be described has been obtained.

Briefly, the negative potential drift is balanced by passing into the medium a controlled stream of very dilute oxygen in nitrogen. The concentration and rate of flow of the gas mixture are adjusted until there is equilibrium between the rate of reduction of the broth system and its rate of oxidation by the oxygen; the potential then remains constant.

The advantage of using oxygen lies in its comparative inertness as an oxidising agent, the ease and smoothness with which the rate of addition can be controlled, and the fact that no solvent etc. accumulates in the medium under examination.

Method.

To obtain a stream of very dilute oxygen advantage is taken of the small quantity of the gas (about 0·5 %) occurring in commercial cylinder nitrogen. In the previous paper a method for completely freeing the nitrogen from oxygen by passage over reduced copper gauze at red heat was described. For the present purpose the nitrogen gas-line to the electrode vessels is modified so as to by-pass a proportion of the stream of impure nitrogen before the copper gauze de-oxygenator (Fig. 1). Flowmeters (FM_1 and FM_2) are included in each branch of the gas-line so that the volume of gas passing through the purifier and that by-passed can be measured.

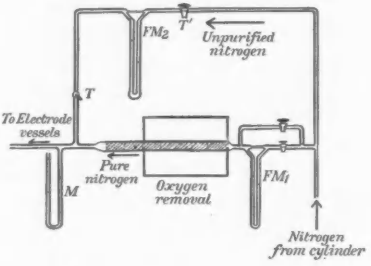

Fig. 1.

The manometer (M) measures the internal pressure at entry to the electrode vessels. It is used in controlling the gas supply to the individual electrode vessels. The by-pass to flowmeter FM_1 is a convenience to prevent a sudden

rush of gas blowing over the liquid in the flowmeter on first opening the cylinder valve. It is afterwards closed.

A diagrammatic curve showing the stabilising of potential is given in Fig. 2 to which the letters in brackets refer. The technique which has been

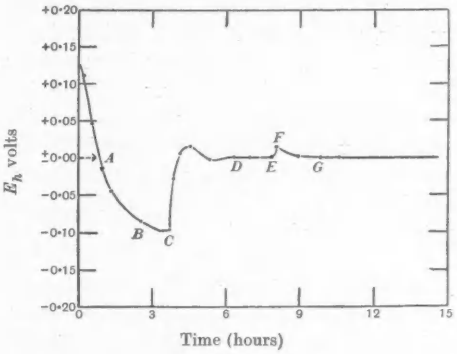

Fig. 2. Diagrammatic curve illustrating the stabilisation of O/R potential of sterile broth (p_H 7·6) at E_h 0·00 volt.

found most satisfactory in practice is as follows. The electrode vessel and its contents are first de-aerated by a fairly vigorous stream of pure nitrogen with by-pass taps *T* and *T'* shut. The potential is allowed to drift negative to the value at which it is ultimately desired to poise it (*A*). Potential readings are made every half-hour. When the potential is sufficiently negative (*B*), the gas rate is reduced at the supply cylinder to give a suitable degree of stirring. The by-pass taps, *T* and *T'*, are then slowly opened to give a small flow of unpurified gas (*C*). Experience will tell what the by-pass flowmeter readings should be, for a given volume of medium, to give approximately the required potential level. Once the medium has been thoroughly de-aerated, and the electrodes have been in contact with it for several hours, small changes in the degree of reduction of the broth system are relatively rapidly recorded by the electrodes. With gradual alterations of the relative rates of purified and unpurified gas any desired potential level can be obtained with as great precision as the normal differences of the electrode readings will allow. When the gas rates are suitably adjusted potential readings are taken for another hour to make sure that equilibrium is attained (*D*). Provided the gas supply from the cylinder is adequate and the copper purifier is not exhausted, the apparatus may be left running for several days. The potential remains constant if the broth is sterile, or if there is no growth of the micro-organism being examined.

When the potential is constant, inoculation of spores or bacteria can be made (*E*) through a hole in the bung of the electrode vessel, normally plugged with a glass rod. If the volume of the inoculum is small, only a very slight temporary change of potential takes place (*F*), and the former level is regained

after about half an hour (*G*). Samples of the medium may be pipetted off similarly.

Poising in presence of oxidation-reduction dyes.

The general behaviour of electrodes in broth has been described. When the oxygen method of poising is used the agreement between electrodes is of the same order, namely, a difference of 10 mv. or less. In prolonged experiments, 12 hours or more, the agreement tends to become closer. Much greater precision can be given to the electrode readings, and the poising of the potential is made more refined, by the addition of small quantities of O/R dyes. A dye is chosen whose span of potential covers the desired level. The dye potentials then control the electrodes, and the latter agree to within 1 mv.

In this case the dye acts as an "indicator" of the potential of the broth system and the potential of the latter is recorded at the electrode *via* the dye potential.

In any particular case it must be shown that there is no specific action by the dye. It is possible, for instance, that a dye might be toxic to a particular organism. In the absence of any specific effect, the addition of small quantities of dye has the further advantage that, with care, a simple potentiometer circuit can be used in the measurement of potential level, at least in orientating experiments.

Span of potential.

Commercial cylinder nitrogen contains about 0·5 % oxygen. Using 0 % to 100 % of unpurified gas mixed with oxygen-free nitrogen the range of potentials between the lowest value to which a medium will drift and a potential of about $E_h + 0{\cdot}175$ volt at p_H 7, can be covered by suitable alteration of proportions of the two gases and of the rate of flow through the electrode vessel. The positive limit at which it is possible to work with these gas mixtures is rather indefinite, but is more positive the lower the p_H. This is to be expected from the general slope of the E_h–p_H curve [Knight, 1930, Fig. 2].

It may be necessary to use a small flow of air (from a cylinder of the compressed gas) instead of by-passing unpurified nitrogen, if it is desired to poise in a zone positive to about $E_h + 0{\cdot}25$ volt.

The electrodes do not behave well positive to a zone at about $E_h + 0{\cdot}175$ volt, in the absence of O/R dyes. By analogy with the behaviour of electrodes in O/R dye solutions, this is probably due to the almost complete oxidation of the systems responsible for the broth potentials. In the presence of a suitable dye or other reversible system it would be possible to work at high levels with as great ease as at the lower levels.

No attempt has been made to correlate actual partial pressures of oxygen and velocities of flow with the O/R potential obtained in any sample of medium, although in bacteriological literature it is customary to correlate "degree of anaerobiosis" with partial pressure of oxygen. If O/R potential is taken as the index of degree of anaerobiosis there is no necessary relation

between oxygen pressure and bacterial growth. The pressure of oxygen capable of being tolerated by an anaerobe will vary with the reducing intensity and capacity of the medium.

While the above method of poising has at present only been used with bacteriological culture media, it could obviously be applied to any other system which exhibited a negative potential drift with time, for example, cell and tissue suspensions.

SUMMARY.

A method has been described whereby the oxidation-reduction potential of bacteriological culture media can be held constant.

The expenses of this investigation were defrayed by the Government Grants Committee of the Royal Society and the Yarrow Research Fund of the London Hospital.

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CXXIV. ZWITTERIONS.

I. PROOF OF THE ZWITTERION CONSTITUTION OF THE AMINO-ACID MOLECULE.

By LESLIE JULIUS HARRIS[1].

II. AMINO-ACIDS, POLYPEPTIDES, ETC., AND PROTEINS AS ZWITTERIONS, WITH INSTANCES OF NON-ZWITTERION AMPHOLYTES.

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(*Received June 30, 1930.*)

Part I.

The suggestion that amino-acids might possess the zwitterion structure (1), $^{+}NH_3 . R . COO^{-}$ (rather than the classical structure (2), $NH_2 . R . COOH$), put forward by Adams [1916] and Bjerrum [1923], was based on a consideration of the acid and basic strengths of related compounds. Derivatives of acetic acid, etc., on the one hand, or of certain amines, etc., on the other, possessed fairly strongly acid or basic properties; and it seemed reasonable therefore to assign similar strongly acid and basic properties to glycine; which is indicated by formula (1) above (in opposition to formula (2), with its weak acid and basic properties). However, notwithstanding the force of the arguments adduced by Bjerrum, the zwitterion theory failed to receive any general acceptance, and Michaelis [1926] only reflects the almost universal view when he concludes that if any zwitterion is present it can be only "in extremely small amounts." As Michaelis cogently points out [1926], "There is no direct method up to the present time of demonstrating concretely the existence of the zwitterion...," for the methods adopted in the past for determining dissociation constants (titration curves, conductivity measurements, etc.) have been of no avail for deciding which view is correct. In the present communication[2] I am, however, able to bring forward direct evidence to show that the amino-acid molecule does possess, in preponderating proportion, the zwitterion and not the classical structure.

The implications of the new view are more far-reaching than may be apparent at first sight—the whole conception of the effects of acids and alkalis

[1] In the whole-time service of the Medical Research Council.

[2] Read before the Biochemical Society on Oct. 6th, 1928 [Harris, 1928].

on the ionisation of ampholytes has to be reversed; and new formulae must be attached to the ampholytes, and also to certain of their salts.

Effect of adding HCl and NaOH to ampholytes: implications of the zwitterion theory.

According to the classical theory addition of HCl to an amino-acid was supposed to cause ionisation of the basic NH_2-group as hydrochloride, and addition of NaOH was supposed to cause ionisation of the acid COOH-group as sodium salt. (The first of these reactions afforded a measure of k_b, the apparent basic strength of the ampholyte; and the second a measure of k_a, the apparent acid strength of the ampholyte[1].)

$$Cl^- + {}^+NH_3 . R . COOH \xleftarrow[(k_b;\ \text{low})]{+\ HCl} NH_2 . R . COOH \xrightarrow[(k_a;\ \text{low})]{+\ NaOH} NH_2 . R . COO^- + Na^+ \quad ...(1)\ \text{old view},$$

$$Cl^- + {}^+NH_3 . R . COOH \xleftarrow[(K_A;\ \text{high})]{} {}^+NH_3 . R . COO^- \xrightarrow[(K_B;\ \text{high})]{} NH_2 . R . COO^- + Na^+ \quad ...(2)\ \text{new view}.$$

If, on the other hand, the *zwitterion* formula be correct additions of HCl and NaOH have just the reverse effect. For the amino-group of the free amino-acid (centre of equation (2)) is already ionised, so that addition of HCl merely serves to depress the ionisation of the carboxyl group, *i.e.* the acid carboxyl group is "replaced" by the stronger acid, HCl. And similarly with the addition of NaOH—the NaOH being a stronger base "replaces" the weaker base, *i.e.* the amino-group; and the ionisation of the carboxyl-group is left unchanged. (According to the new view, then, the addition of HCl measures the acid dissociation constant instead of the basic constant of the old view. Similarly the addition of NaOH measures the basic constant; for this addition does not affect the acid dissociation, but it depresses the basic dissociation.)

Interpretation of the HCl and NaOH titration curves of glycine according to the old and new theories respectively.

The above argument means, in brief, that the dissociation constants must be reversed if the new view is true: what was described as the basic constant according to the old view now relates to the new acid constant, and *vice versa*. The amino-acid is no longer considered as a potential weak base and a potential weak acid, but a comparatively strong acid and base simultaneously.

If we examine the corrected [Harris, 1923, 1; 1925, 1] titration curve [Henderson, 1908; Hasselbalch, 1911] of a comparatively weak acid, A_4B_4 (Fig. 1), we see that it is identical with the back titration curve of a comparatively strong base, $A_4'B_4'$. Similarly, the titration curve of a comparatively weak base $B_1'A_1'$ is indistinguishable from the back titration (replacement) curve of a comparatively strong acid B_1A_1. In general, titration curves of bases are indistinguishable from back titration curves of acids and the back

[1] For simplicity we shall use ionisation, instead of activity, notation throughout the present paper.

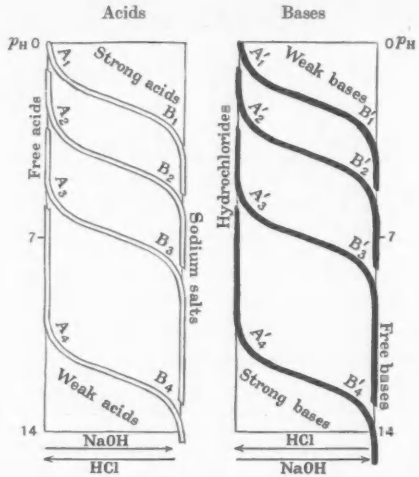


Fig. 1. Identity of titration curves of strong (weak) acids with back titration curves of weak (strong) bases, and *vice versa*. ("Corrected" titration curves.)

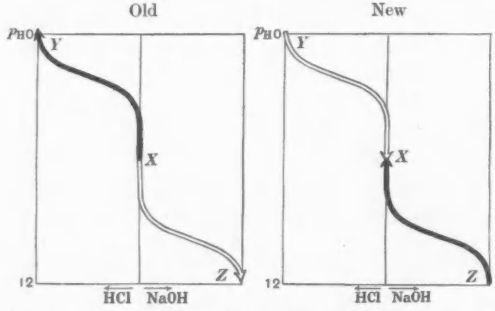


Fig. 2. Alternative interpretations of the experimental titration curve of glycine. (Position of arrow on curve shows end-point.)
═, acid group ▬, basic group.

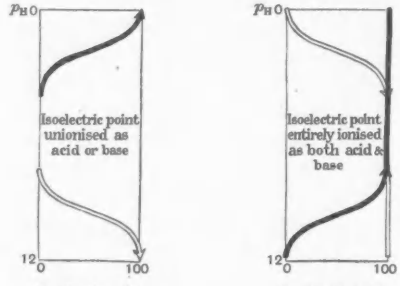


Fig. 3. Ionisation curves of glycine according to (A) new, and (B) old, views.

titration curves of bases from the titration curve of acids; in fact they coincide when the p_K (= p_H mid-point) is the same.

In the case of glycine, the experimental titration curve [Harris, 1923, 1] is shown in Fig. 2. This may be interpreted in two different ways, and we are unable to say by inspection of the curve which is correct. Either (1) XY is the titration curve of the basic amino-group and XZ is the titration curve of the acid carboxyl group, or (2) XY is the replacement curve of the acid carboxyl group and XZ is the replacement curve of the basic group. The first alternative implies that the amino-acid possesses the classical structure, and the second the zwitterion structure. If the first alternative is true, ionisation is at a maximum at highly acid and alkaline p_H values, and at a minimum at the isoelectric point. If the second alternative is true, on the contrary, ionisation is at a maximum at the isoelectric point (simultaneously as acid and base) (Fig. 3).

In earlier papers these two alternatives were recognised, but it was pointed out that it was immaterial from a practical point of view, in carrying out titrations for estimating acid and basic groups, which view was correct. With no effect on the practical accuracy, one could define the HCl curve (XY), either by the symbol k_b for an apparent basic group, or by the symbol [Bjerrum, 1923] K_A for the back titration of an acid group; and similarly the NaOH curve XZ, might be defined by an apparent acid constant, k_a, or by a zwitterion basic constant K_B [Harris, 1923, 1, p. 441; 1925, 2, p. 381].

If one could discover whether XY is a basic titration curve or an acid back-titration curve (or alternatively, whether XZ is an acid titration curve, or a basic back-titration curve) one would be in a position to decide which of the two theories is correct, classical or zwitterion.

Practical.

According to the new view, the amino-acid solution may be compared with a solution of ammonium acetate (*i.e.* moderately strong base plus moderately strong acid). According to the old view it may be compared with a solution of very weak base and very weak acid (*e.g.* aniline plus boric acid). That is, in the first instance the HCl and NaOH curves are back titration curves, of acid and basic groups respectively (*i.e.* acetate replaced by HCl and ammonium by NaOH); in the second they are direct titration curves of basic and acid groups respectively (*i.e.* aniline converted to hydrochloride, and boric acid to sodium salt)[1].

$$\underset{\mathbf{K_a}}{\overset{+\,HCl}{\longleftarrow}} \qquad \underset{\mathbf{K_b}}{\overset{+\,NaOH}{\longrightarrow}}$$

$$NH_4^+ + CH_3COOH \longleftarrow NH_4^+ + CH_3COO^- \longrightarrow NH_3 + CH_3COO^-$$

$$C_6H_5NH_3^+ + H_3BO_3 \longleftarrow C_6H_5NH_2 + H_3BO_3 \longrightarrow C_6H_5NH_2 + H_2BO_3^-.$$

$\mathbf{K_b}$ $\mathbf{K_a}$

The technique of the present study involves the determination of acid-base combination curves (corrected titration curves) in the presence of form-

[1] In the case of the amino-acid, the two charges are of course carried by the same molecule; in the analogues cited by separate ions.

aldehyde, and a comparison of the results so obtained with those in water. Experimental details of the method have already been described at length [Harris, 1929].

Results.

When various carboxylic acids are titrated in dilute formaldehyde it is found that there is, in general, no significant shift in the position of the titration curve compared with that in water. On the other hand, when nitrogenous bases such as ammonia and amines are so titrated there is, in general, a very considerable shift of the titration curve in the direction of diminished basicity, owing to the formation of more weakly basic compounds of the type of methylene derivatives in equilibrium with the original base. The extent of the shift depends on the relative excess of formaldehyde present. We therefore find a very wide generalisation that a curve which shows a marked shift to diminished basicity, in presence of formaldehyde, relates to a basic group, and that acid groups are not so shifted.

Experimental results with ammonium acetate (0·1 *N* sol.) are shown in Fig. 4. Here the NaOH curve *XZ* is already known to relate to the basic

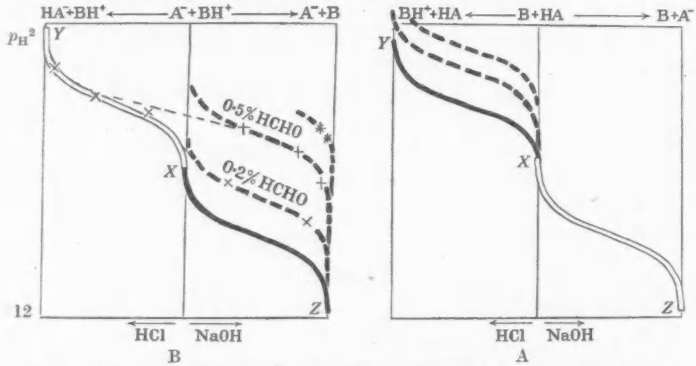


Fig. 4. Effect of small additions of formaldehyde on titration curves of (A) aniline and boric acid, (B) ammonium acetate.
——, acid curve. — — —, basic curve. — — —, basic curve in HCHO.

ion (p_K for ammonia being 9·3) and HCl curve *XY* to the acid ion (p_K for acetic acid being 4·7). As anticipated, the HCl curve is not appreciably shifted (since it relates to an acid group); and the NaOH curve is very considerably shifted (since it relates to a basic group). In the case of a weak acid-weak base mixture (analogue of classical ampholyte formula) the reverse is the case. The HCl curve, as expected, is shifted since it relates to the basic group, and the NaOH curve, which related to the acid group, is comparatively unaffected[1].

[1] With certain weak acids (SH, and boric acid) it is possible to detect the existence of a reaction with formaldehyde and some resulting shift in p_k value. In the case of boric acid, the shift is still relatively slight even in high concentrations of formaldehyde, and so remains in contrast with the large shift shown by bases in quite low concentrations of formaldehyde. With SH the shift is in the opposite direction to that of a base (see Part II, Fig. 18).

The result with glycine is shown in Fig. 5. The full experimental values for this and five other amino-acids have already been given elsewhere, where the conclusions here adduced were briefly summarised, and the analytical applications discussed in detail [Harris, 1929]. As was earlier predicted would be the case [Harris, 1923, 2], there is a considerable increase in k_a (= decrease in K_B), the constant for the apparent acid group, titratable by NaOH [Harris, 1925, 2]. The HCl curve, on the other hand, is not appreciably changed.

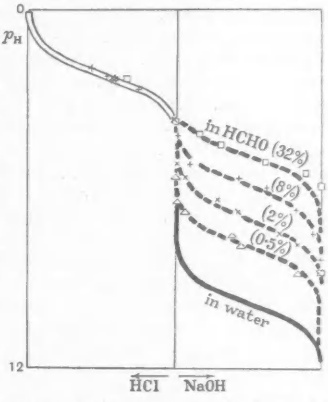


Fig. 5. Titration curves of glycine in presence of increasing concentrations of HCHO.

□, 32 % HCHO. +, 8 % HCHO.
×, 2 % HCHO. △, 0·5 % HCHO.

The fact that the NaOH curve is shifted towards diminished basicity, in the manner characteristic of basic groups, and that the HCl curve remains virtually unaffected, as is customary with acid groups, forces one to the conclusion that the former is the basic (back titration) curve and the latter the acid (back titration) curve, *i.e.* that the amino-acid has the zwitterion and not the classical constitution. Any other conclusion would involve ridiculous assumptions. The alternative interpretation, that there is an immense increase in the acid strength and virtually no measurable change in the basic strength, would obviously be meaningless. The formaldehyde is, in fact, added with the express purpose of combining with the NH_2-group and weakening its basicity, a property which is made use of in elementary analytical operations. And it would be equally ludicrous to imagine that so great an increase (*e.g.* ten-thousand fold) in the real strength (as opposed to k_a) of the carboxyl group could result from a slight modification in, maybe, a distant amino-group[1]. The entire parallel between the amino-acid and the zwitterion-analogue (ammonium acetate) and the unmistakable contrast between the amino-acid and the classical analogue (weak acid plus weak base) in their reactions to titration in formaldehyde, form sufficiently striking evidence in themselves.

Additional confirmation will be apparent (1) if it is shown for a compound containing x amino-groups and y carboxyl groups, that x of its curves are shifted by formaldehyde, and y remain virtually stationary; and also (2) if examples of certain ampholytes predicted to be of the classical and not of the zwitterion type are examined, and it is shown that here, in contrast with glycine, there is a shift not of the NaOH but of the HCl curve. Such confirmation is given in the next section.

[1] *E.g.* in the case of polypeptides (see Part II) where the distance of the amino- from the carboxyl group precludes the possibility of such an influence.

Part II.

Technical details. The technique was the same as that described earlier except that instead of making a separate colorimetric determination for each fresh point we used the hydrogen-electrode (in conjunction with a calomel-half-cell) for determining continuous p_H curves. With the exception of arginine and histidine which were titrated at 23°, and *p*-aminophenol at 25°, all titrations were carried out at room temperature, 14–19°. E.M.F. was measured by a Cambridge-Paul portable potentiometer. Our absolute p_H standard was 0·1 *N* HCl ($p_H = 1·07$) and the calomel cell and potentiometer were standardised against it at frequent intervals. In practically all cases titrations were carried out at a concentration of 0·1 *M*, either 2 or 5 cc. of the solution being used. In special cases, namely tyrosine, *p*-aminobenzoic acid and glycyltryptophan, concentrations as dilute as 0·01 *M* had to be used owing to the insolubility of the ampholyte and its methylene derivative at the isoelectric point. Glycyltryptophan behaved peculiarly: its methylene derivative although soluble on the alkaline side of the isoelectric point appeared to be practically insoluble on the acid side even in high concentrations of HCl.

In order to avoid any appreciable change in volume during titration *N* HCl and NaOH were used, being added from a micro-burette graduated in 0·01 cc. To save the necessity of performing two separate titrations, the requisite number of equivalents of acid or alkali were added to the free amino-acid so as to bring the p_H to the extreme end of either the acid or the alkali curve, and a single back titration was then carried out with either alkali or acid. Small concentrations of formaldehyde, 0·25–2 %, were found to be sufficient for obtaining an appreciable shift of the basic curve. The presence of formaldehyde in such concentration did not appear to affect the working of the hydrogen-electrode to any extent—since hardly any change in E.M.F. (1 m.v. or less) was observed when a small volume of water which had been brought to a given p_H was mixed with large excess of 5 % formaldehyde at the same p_H. Approximate blank corrections were made by determining the amount of acid or alkali required to bring the same volume of aqueous or dilute formaldehyde solvent to the same p_H in absence of the solute. The blank correction on the acid side is practically the same for dilute formaldehyde as for water. On the alkaline side, however, the blank for formaldehyde becomes considerable above p_H 10·5. Strictly speaking, the blanks obtained in this way are slightly too large, for the amino-acid uses up an appreciable proportion of the formaldehyde to form the methylene derivative.

In the case of *p*-aminobenzoic acid, the HCl curve has its p_k at a highly acid reaction (p_H 2) and the extreme insolubility of the methylene derivative necessitated the use of a large volume of solvent, so that there was a very large blank correction, and it became difficult to measure accurately the extent of the shift in formaldehyde. To overcome this difficulty, a 0·1 *M* solution of the ampholyte was made up with sufficient HCl to bring it to p_H 1·75, 18 cc. of a 5 % solution of formaldehyde were brought to the same p_H, and then 2 cc. of the ampholyte solution were added. The p_H dropped to 1·42. The amount of NaOH required to bring the p_H back to 1·75 was then determined (see Fig. 16). Controls were done by adding 2 cc. of ampholyte to 18 cc. of H_2O at p_H 1·75, and by diluting 18 cc. of 5 % formaldehyde with 2 cc. of H_2O at the same p_H. No appreciable shift in p_H was observed in either case. Similar points were determined on the NaOH curve. The same method was used with *p*-aminophenol.

The gelatin curve was obtained as follows. 5 cc. of a 1 % solution of gelatin were taken for each point and varying amounts of acid or alkali were added to each sample. In the presence of formaldehyde two of the p_H values (7·4 and 7·65) were determined colorimetrically because the formation of a gel interfered with the ready use of the hydrogen-electrode. The first NaOH curves for tyrosine and aspartic acid in presence of 16 % formaldehyde were also done colorimetrically as previously described [Harris, 1929].

1. Dibase-monoacids (histidine, lysine, arginine).

Figs. 6 and 7 show the results with histidine and lysine. It will be seen that two curves are considerably shifted and one remains virtually stationary, in the anticipated manner. The fact that the NaOH curve is shifted implies

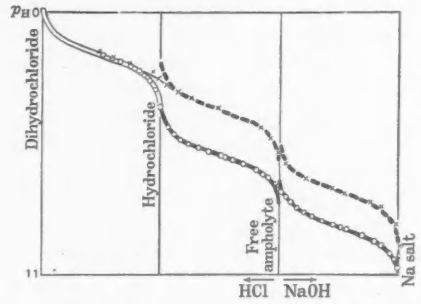


Fig. 6. Titration curves of histidine, in water and in HCHO.
○, in water. ×, in HCHO (1 %).

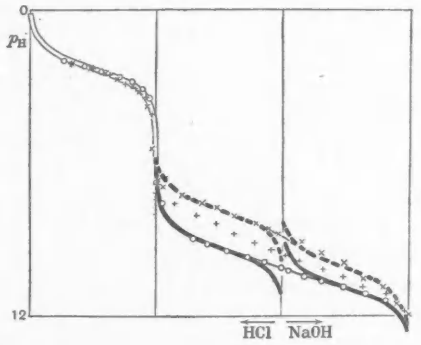


Fig. 7. Titration curves of lysine, in water and HCHO respectively.
○, in water. +, in HCHO (1 %). ×, in HCHO (2 %).

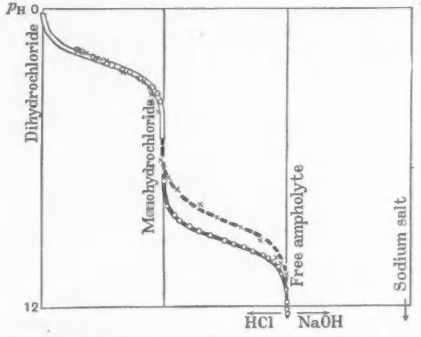
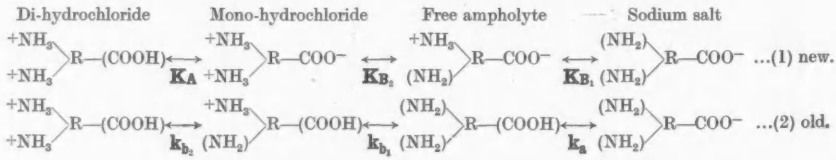


Fig. 8. Titration of arginine in water and HCHO.
○, in water. ×, in HCHO (0·25 %).

that histidine and lysine possess the zwitterion structure. Therefore the addition of NaOH does not represent neutralisation of the carboxyl-group as formerly supposed, but back titration of the stronger basic group. The first addition of HCl causes the ionisation as hydrochloride not of the stronger, but of the weaker of the two basic groups; while addition of the second equivalent of HCl contrary to former belief, represents not neutralisation of the weaker basic group but back titration of the fairly strong acid (COOH) group. Similar results are obtained with arginine except that the guanidine group is so strongly basic that its titration curve occurs in an inaccessibly alkaline zone and that the determination of a shift is rendered difficult by the very large blank correction.

Equation (1) shows the new view of the compounds formed when HCl and NaOH are added to these ampholytes; equation (2) representing the old view. (Addition of HCl, right to left; addition of NaOH, left to right.)



2. Monobase-diacids (aspartic acid, glutamic acid, tyrosine, cysteine, Figs. 9, 10 and 11).

In the case of aspartic and glutamic acids (Fig. 9), we have one curve showing the typical shift and two virtually stationary, corresponding with one NH_2- and two COOH-groups. (Tyrosine and cysteine (Figs. 10 and 11) with their very weakly acid OH- and SH-groups are similar, apart from the distinctive behaviour of the SH-group towards formaldehyde[1].) Addition of HCl represents not titration of NH_2 as in the old view, but back titration of a fairly strongly acid COOH-group, viz. the stronger of the two COOH-groups in glutamic and aspartic acid. Addition of the first equivalent of NaOH represents, in the cases of glutamic and aspartic acids, titration of the weaker of the two COOH-groups and not the stronger, as in the old view; in the cases of tyrosine and cysteine, it represents back titration of the NH_2-group, instead of titration of the stronger COOH as in the old view. Addition of the second equivalent of NaOH represents, in the cases of glutamic and aspartic acids, back titration of the fairly strong NH_2-group, in place of titration of the very weak second COOH-group on the old view; in the cases of tyrosine and cysteine it no doubt represents titration of the very weak OH- and SH-groups respectively[1].

Cannan and Knight [1927], it will be recalled, were unable to decide which

[1] It will be seen (Fig. 11) that there is evidence of a reaction occurring between the SH-group of cysteine and HCHO which results in a perceptible weakening of its acidity and a measurable shift of the p_k to increased basicity [cf. Pirie and Pinhey, 1929] very distinct from the typical shift to diminished basicity characteristic of a basic group.

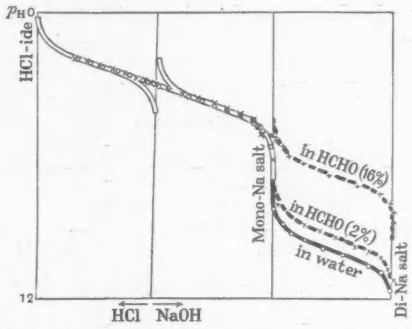


Fig. 9. Titration of aspartic acid in water and HCHO.
○, in water. ×, in HCHO (2 %). +, in HCHO (16 %).

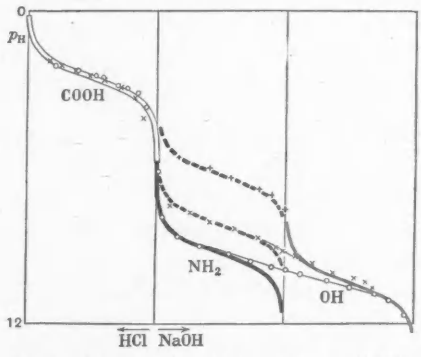


Fig. 10. Titration of tyrosine in water and HCHO.
○, in water. ×, in HCHO (2 %). +, in HCHO (16 %).

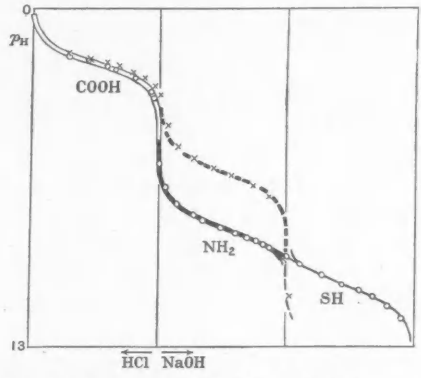
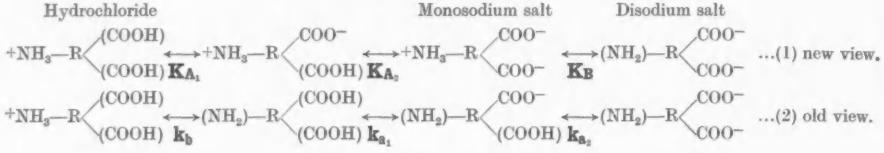


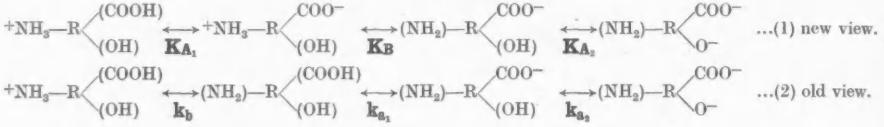
Fig. 11. Titration of cysteine in water and HCHO. ○, in water. ×, in HCHO (1·5 %).

arrangement was probable for cysteine, since, with the evidence then available "plausible argument could be made for either allocation."

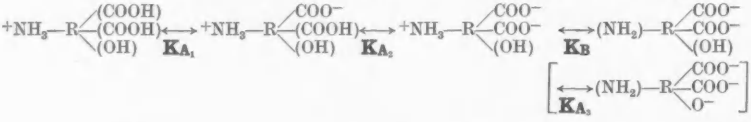
For glutamic and aspartic acids.



For tyrosine (and cysteine)[1].



For hydroxyglutamic acid (not yet examined), the reactions may be predicted as follows, with a vanishingly small K_{A_3}:



It will be seen that new formulae are given for the monohydrochloride and the free amino-acid, in the case of the monoacid-dibasic ampholytes; the dihydrochloride and sodium salt retaining the old constitution. With glutamic and aspartic acids, the monosodium salt and free ampholyte are given new constitutions, the other salts remaining unchanged; but with tyrosine and cysteine, only the free amino-acid is changed and both the sodium salts are unaltered.

3. Polypeptides as zwitterions.

In Figs. 12 and 13 are shown typical results with a di- and a tri-peptide, glycyltryptophan, and glycylglycylglycine. It will be observed that these compounds behave similarly to the simple amino-acids with the same number of basic and acid groups from which they are derived. The constants of peptides show [Harris, 1923, 1] a slight decrease in value of K_A and K_B compared with the corresponding amino-acid as, of course, is to be anticipated, from the removal of the influence of the neighbouring ionised group. The constants of glycyltryptophan not hitherto known are reported elsewhere [Birch, 1930].

[1] For cysteine put SH for OH in equations following.

4. Taurine.

The constants of taurine in themselves afford very suggestive evidence, by analogy, for the zwitterion theory. Taurine contains one amino-group,

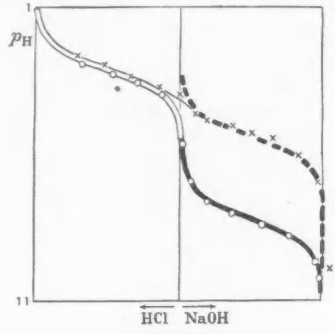


Fig. 12.

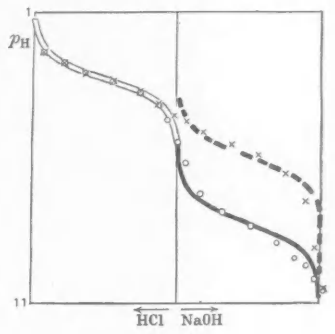


Fig. 13.

Fig. 12. Titration curve of a dipeptide, glycyltryptophan, in water and HCHO.
○, in water. ×, in HCHO (1 %).

Fig. 13. Titration curve of a tripeptide, glycylglycylglycine, in water and HCHO.
○, in water. ×, in HCHO (1 %).

therein resembling an ordinary α-monoaminomonocarboxylic acid; but the usual carboxyl group is replaced by sulphonic acid. According to the classical view the acid dissociation constant of taurine is quite small, corresponding in fact with that of other amino-acids; but the basic dissociation constant is vanishingly small. This view is from the first difficult to accept, because one would expect a very high acid constant as in other sulphonic acids, and a basic constant similar to that of an ordinary α-amino-acid or peptide.

The constants fall into line, however, if the new view is accepted, K_A being very high, as in other sulphonic acids; and K_B being of the same order as K_B for analogous compounds (*i.e.* fairly high). The NaOH curve, that is, is presumed to be the displacement curve of the NH_2-group and not the titration curve of the sulphonic acid group.

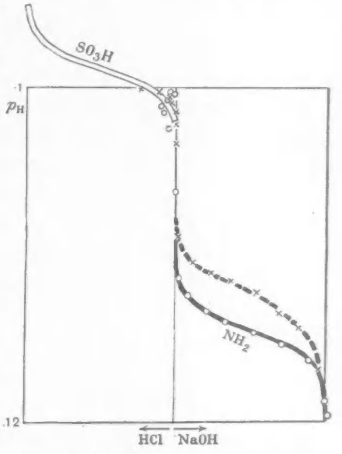


Fig. 14. Titration curves of taurine as *experimentum crucis*.
○, in water. ×, in HCHO (2 %).

Our formaldehyde titration curve of taurine (Fig. 14) confirms very decisively the zwitterion hypothesis. The NaOH curve is markedly shifted, in the manner

characteristic of a base or the basic groups of the zwitterion amino-acids. It would be impossible to picture formaldehyde causing a very marked increase in the acid constant of the sulphonic acid group, which would be the alternative interpretation.

5. Demonstration of non-zwitterion constitution of certain ampholytes.

Interpreting the glycine titration curve in the zwitterion fashion, as we are now entitled to do, the acid and basic curves are seen to fall in the same relative positions as in the combined curves of acetic acid plus ammonia (*i.e.* the HCl curve refers to the acid group, and the NaOH curve to the basic group)[1] (see Part I, Figs. 2 and 4). When, in contrast, we consider the combined curves of a weak base such as aniline and a weak acid such as phenol we see that they possess, to an extreme degree, the formal appearance of the predicted curves of the non-zwitterion type of electrolyte (that is, the HCl is the basic curve and the NaOH the acid curve). Hence we are led to suppose that the ampholyte derived from these two substances, that is aminophenol, will have the non-zwitterion form (Fig. 15). To test the accuracy of our supposition, we have determined the titration curves of *p*-aminophenol in water and in formaldehyde. The result confirms our prediction and definitely establishes the non-zwitterion nature of this ampholyte. For, in direct contrast with glycine, the HCl curve shows the characteristic displacement to a lowered p_H, and the NaOH curve is virtually unchanged. Hence, the HCl curve

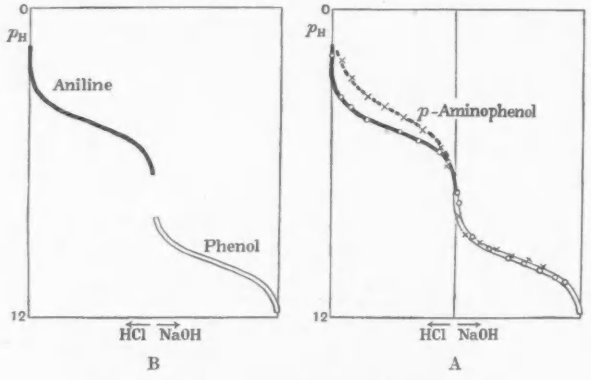


Fig. 15. Titration curves (A) of *p*-aminophenol and (B) of parent substances, aniline and phenol.
○, in water. ×, in HCHO (1·5 %).

[1] For a quantitative prediction of the precise p_k values it would be necessary to pay regard to the electrostatic effect of the simultaneously ionised neighbouring groups; and the ampholyte is more accurately to be compared not with the parent substances themselves, acetic acid and ammonia, but with their substituted derivatives.

relates to the basic, and the NaOH curve to the acid group. The action of HCl and NaOH is to be represented

$$^{+}NH_3 . R . (OH) \xleftarrow{HCl} (NH_2) . R . (OH) \xrightarrow{NaOH} (NH_2) . R . O^{-}.$$

p-*Aminobenzoic acid.* In the case of *p*-aminobenzoic acid the comparative weakness of the parent substances (aniline and benzoic acid) again renders the non-zwitterion structure more likely. In this instance, however, the prediction might at first sight seem somewhat less secure, because benzoic acid is by no means so weak an acid as is phenol in the case just cited. The known constants for *p*-aminobenzoic acid are 2·0 and 4·8. If 2·0 is the basic and 4·8 the acid constant, *p*-aminobenzoic acid will be a non-zwitterion. If, conversely, 2·0 is the acid and 4·8 the basic constant, *p*-aminobenzoic acid will be a zwitterion. The constants of the parent substances benzoic acid and aniline are

$$\text{benzoic acid, } p_k \quad \ldots \quad = 4{\cdot}2,$$
$$\text{aniline, } p_k \quad \ldots \quad \ldots \quad = 4{\cdot}6;$$

a combination of which values would represent a bare border-line case of zwitterion. However, it is more accurate to take as types not the parent substances, benzoic acid and aniline, but comparable *para*-substituted derivatives. We are indebted to Mr J. B. S. Haldane for the suggestion that *p*-aminobenzoic acid may be predicted to have an acid constant near to that of *p*-methyl (or hydroxy)-benzoic acid and a basic constant near to that of *p*-nitroaniline. The values in question are

$$p\text{-nitroaniline, } p_k \quad \ldots \quad \ldots \quad \ldots \quad = 2{\cdot}0,$$
$$p\text{-methyl (or hydroxy)-benzoic acid, } p_k = 4{\cdot}4\text{–}4{\cdot}5.$$

That is, the predicted values of *p*-aminobenzoic acid are very close to the known values, provided the latter be taken in the non-zwitterion order. Our experimental results appear to indicate that *p*-aminobenzoic acid does indeed possess the non-zwitterion form, to a preponderating extent. For we find that the HCl curve is shifted by HCHO in the manner typical of a basic group and the NaOH curve is not significantly changed (Fig. 16).

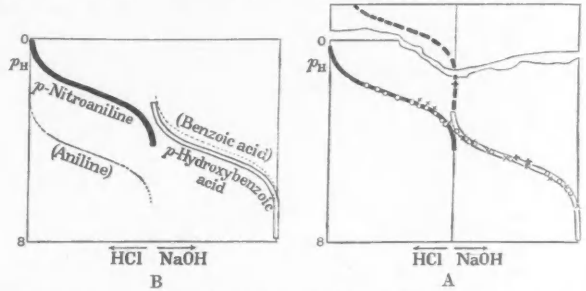
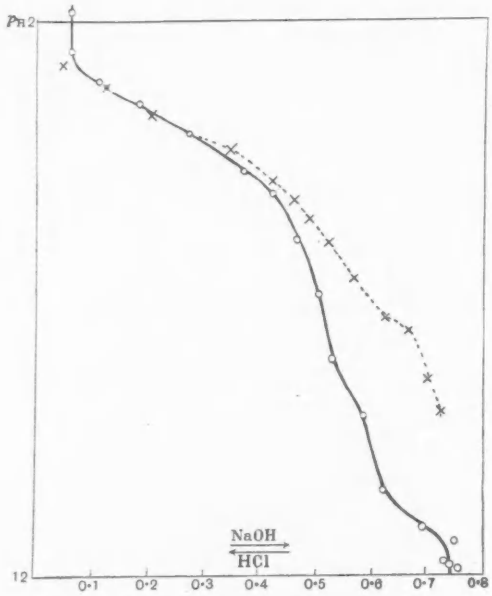


Fig. 16. Titration curves of (A) *p*-aminobenzoic acid in water and in HCHO and (B) combined titration curves of parent substances and related derivatives.
○, in water. ×, in HCHO (0·5 %). +, in HCHO (5·0 %).

6. Proteins as zwitterions.

Having shown that amino-acids possess the zwitterion structure and then extended our observation to embrace di- and tri-peptides, it became almost a foregone conclusion that proteins also would be found, when we came to apply the same technique, to belong to this category. In the case of one protein, gelatin, we have already pointed out [Harris, 1925, 2] that it was impossible to accept the previously universal view that the isoelectric molecule possessed no ionic charges, because the p_H difference between the acid end-point at p_H 2·4 and the isoelectric point at p_H 4·7 is less than the minimum of 4 p_H units, which is necessary to cover the conversion (98 %) of even one charged group into its non-charged state. Therefore, since isoelectric gelatin possesses no net charge, positive or negative, it was necessary to assume that there was an equal number of negative charges to balance the positive, unless, of course, the accepted values for the acid end-point and isoelectric point could be shown to be in error. But the foregoing argument, while demonstrating that isoelectric gelatin is no longer to be regarded as non-ionised, does not go so far as to indicate that the titration of the principal acid groups occurs in the acid p_H range and that of the principal basic groups in the alkaline range, which



Cc. of $N/1$ acid or alkali per 0·5 g. crude protein

Fig. 17. Titration curves of gelatin in water and in HCHO.
○, in water. ×, in HCHO (1 %).

is the full implication of the zwitterion results in the case of amino-acids and peptides. This we now show to apply to gelatin by our formaldehyde titrations which are summarised in Fig. 17. The ionisation of gelatin is therefore to be represented mainly as

$$(^{+}NH_3)_x \cdot Pr \cdot (COOH)_y \xleftarrow{+\ HCl} (^{+}NH_3)_x \cdot Pr \cdot (COO^{-})_y \xrightarrow{+\ NaOH} (NH_2)_x \cdot Pr \cdot (COO^{-})_y.$$

7. Controls with various acids and bases.

The general behaviour of single bases and acids with HCHO is summarised in Fig. 18. Acetic and other carboxylic acids are not significantly affected. Ammonia, various amines, etc., show considerable shifts with very small additions of formaldehyde. With boric acid a shift can be detected but is comparatively small even with large amounts of formaldehyde.

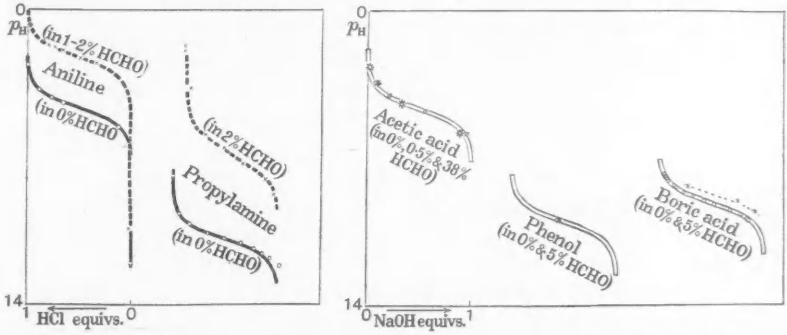


Fig. 18. Effect of formaldehyde on titration curves of various bases and acids.
○, in water. ✱, in 38 % HCHO, colorimetric. ⁎, in 5 % HCHO.
×, in 1–2 % HCHO. /, in 0·5 % HCHO.

8. Addendum on the temperature coefficient method.

Elsewhere [Birch and Harris, 1930] we have referred to a further method which enables us to distinguish between a carboxyl and an amino-group in the titration curve of a given amino-acid, and hence to obtain evidence for or against the zwitterion structure. Briefly it depends on the observation that the p_k of a carboxyl group shows little change with temperature when calculated as an acid constant on the p_H scale, while the p_k of an aliphatic amino-group shows little change with temperature when calculated as a basic constant on the p_{OH} scale. The relative allocation of curves to carboxyl and amino-groups as deduced from this method agrees with the conclusions to be drawn from the formaldehyde titration method; and we have described the application of the method to the identification of the three constitutions of the titration curve of histidine and of arginine.

SUMMARY.

PART I.

The HCl and NaOH titration curves of glycine (measured by p_H indicator virages) have been determined in the presence of increasing concentrations of formaldehyde. The weakening of the basicity of the NH_2-group caused by the formation of a methylene derivative is found to result in a displacement not of the HCl curve (*i.e.* the apparent basic curve), but of the NaOH curve (*i.e.* the apparent acid curve). It follows that the HCl and NaOH titration curves of the amino-acid represent, not neutralisation of NH_2 and COOH respectively, as in the classical view, but "replacement" (back titration) of COOH and of NH_2 by a stronger acid and a stronger base, HCl and NaOH respectively.

Direct evidence is therefore available of the accuracy of the zwitterion (ionised internal salt) theory.

That the shift with HCHO to lowered basicity is characteristic of a basic curve is shown by similar investigations with free bases and ammonium salts, etc., and with other amino-acids and ampholytes.

PART II.

With ampholytes containing more than one NH_2- or COOH-group the number of curves characteristically shifted is shown to be equal to the number of basic groups, and those virtually unaffected to the COOH-groups.

The method has been used for determining whether a given ampholyte possesses the zwitterion or non-zwitterion constitution. By its aid it is also possible to determine which are the basic and which the acid components of the titration curves.

New formulae must be ascribed to the monohydrochlorides and the free amino-acids in the case of diaminomonocarboxylic acids, and to the monosodium salts and the free amino-acids in the case of dicarboxymonoamino-acids. The stages in the ionisation of cysteine, tyrosine, and other amino-acids and their salts are similarly set out.

The apparent acid constant of taurine is demonstrated to relate to the basic group.

p-Aminophenol and *p*-aminobenzoic acid are demonstrated to exist as non-zwitterions, HCHO giving a significant shift of the HCl and not the NaOH curve.

Polypeptides are shown to be zwitterions. Similarly with a protein (gelatin) the COOH-groups titrate in the acid and the NH_2-groups in the alkaline p_H range.

COOH- and NH_2-groups in titration curves may be identified also (and hence the zwitterion theory tested) by a new "temperature coefficient" method which depends on the fact that the dissociation constants of NH_2-groups are found to show only small temperature changes when expressed on

the p_{OH} scale and large changes on the p_H scale, and *vice versa* for COOH-groups.

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CXXV. VARIATIONS IN THE COMPONENT FATTY ACIDS OF BUTTER DUE TO CHANGES IN SEASONAL AND FEEDING CONDITIONS.

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Some little time ago a modified form of the ester fractionation process for the analysis of mixed fatty acids, suitable for the case of fats containing appreciable quantities of volatile fatty acids, was worked out and used to determine the composition of three market samples of New Zealand butter [Hilditch and Jones, 1929]. The results then obtained indicated that the analytical procedure was capable of giving more detailed information on the proportions of the various fatty acids present than had formerly been available and held out the possibility that its employment might yield further insight into the much discussed problem of the influence of oleaginous diet on the composition of butter-fat.

The most serious practical difficulty in a programme of this kind is the lengthy nature of the analyses involved, especially in the case of a fat which includes so wide a range of fatty acids as butter; this makes it difficult to deal with more than relatively few test samples in the course of one series of investigations. In illustration of this it may be said that almost two years have been occupied in the detailed study of the five butter-fats which form the main experimental material discussed in this communication, including, in addition to the determination of the fatty acid compositions of the original fats, an examination of their component glyceride structures, with the fatty acid compositions of the fully saturated glycerides present; an account of this part of the work will be presented later. The main part of the investigation was carried out on four samples of butter-fat (about 7–8 lb. each) collected for us from a herd of ten Shorthorn cows at the National Institute for Research in Dairying, Shinfield.

The four samples of butter consisted of (I), butter from the herd at the end of October, 1928, when the cows were still partly pasture-fed but were also receiving winter diet as below; (II) and (III), butter, in each case, from two cows of the herd fed on a general ration with, in addition, either coconut cake or soya bean cake, these butters being from milk collected about the

middle of March, 1929, after the cows had been feeding for nearly a month on the respective diets; and (IV), butter from milk from the whole herd of ten cows collected at the end of May, 1929, after the cows had been feeding on early summer pasture for about a month.

It is convenient also to refer to the figures obtained for a sample of New Zealand butter-fat (V) which was kindly supplied to us by Dr F. H. McDowall from a herd at the Dairy Research Institute, Palmerston North, New Zealand, feeding on early summer pasture in December, 1928, since this particular sample brings out certain features of the problem which emphasise its complexity and the difficulty of correlating cause and effect.

As a matter of fact, the information resulting from our analyses of butter-fats I–IV showed that other factors than the fatty diet supplied exerted important influences, and certain subsidiary tests bearing on this aspect were carried out later on samples of butter-fat obtained monthly from the same herd of Shorthorn cows at Shinfield during the winter of 1929–1930 and the following spring, covering the periods when the cows were receiving the same diet as that from which butter-fat I was derived (winter conditions) and when the herd returned to spring pasture.

In view of the complex factors involved, we believe that it will be found most convenient to describe our experimental results in the first place and then to discuss them, both as a whole, and also in their relation to the results obtained by previous workers in this field.

Fatty acid compositions of butter-fats I–V.

The details of the feeding, etc., of the cows may first be recorded.

Dealing first with the butters from Shinfield, these were made in each case from sweet unheated cream (without starter) at the National Institute farm.

Butter-fat I.

This was from milk from the herd of ten Shorthorn cows collected on October 28–30, 1928. The cows were still getting some grass and also 50 lb. kale and 6 lb. clover hay with 3–12 lb. concentrates (according to the milk yield); the latter consisted of dried grains three parts, maize germ meal two parts, soya bean meal extracted one part.

Butter-fat II (coconut cake-fed) and butter-fat III (soya bean cake-fed).

Two cows of the herd received the coconut cake diet, and other two cows were given the soya bean cake diet. The milk from which the butter samples were prepared was collected on March 12–15, 1929, and the details of feeding prior to the preparation of the butter were as follows.

Each cow received daily 30 lb. mangolds, 30 lb. silage and 15 lb. hay.

The cows on coconut cake ration received in addition:

	Per cow daily	
	Coconut cake	Mixture B*
From Feb. 5	3 lb.	13 lb.
Gradually increasing coconut cake to Feb. 25. And continued these quantities to March 20	7 lb.	7 lb.

Each cow was thus receiving 7 lb. of coconut cake and 0·8 lb. of soya cake daily from Feb. 26 to March 20.

The cows on soya cake ration received in addition:

	Per cow daily		
	Soya cake	Crushed oats	Mixture B*
From Feb. 12	2 lb.	3 lb.	9 lb.
Gradually increasing soya cake to Feb. 25	4 lb.	7 lb.	3 lb.

From Feb. 26 to March 20 the two cows each received 13 lb. of a mixture containing two parts soya cake, one part flaked maize, one part crushed oats and one part sharps: each cow was thus receiving 5·2 lb. soya cake daily from Feb. 26 to March 20.

Butter-fat IV.

This was prepared from milk from the whole herd after they had been out on early summer pasture for several weeks; the milk was collected on May 26 and 27, 1929.

Butter-fat V (New Zealand pasture-fed).

The origin of this sample has already been described.

For the purposes of chemical investigation the butters were melted, settled, and the fat decanted and filtered through two layers of thick filter-paper (which had previously been kept in the steam-oven for some time at 100°) in order to remove traces of caseinogen and any residual moisture. Some of the general analytical characteristics of the fats were determined with the following results:

Table I.

Butter-fat	I	II	III	IV	V
Sap. equiv.	253·3	242·7	243·3	248·2	242·2
Iodine value	41·3	31·6	34·8	41·6	34·5
Reichert-Meissl value	29·6	29·3	32·3	25·7	30·3
Polenske value ...	1·9	3·2	2·0	2·2	2·3
Kirschner value ...	23·7	23·7	25·5	20·6	24·7
Refractive index $n_D^{60°}$	1·4477	1·4450	1·4455	1·4473	1·4462

The determination of the fatty acids present in these butters was carried out almost exactly according to the scheme described at length in the former communication [Hilditch and Jones, 1929], and, except for one or two further precautions which it has been found desirable to observe, it is unnecessary here to repeat the full description of the procedure, which is briefly as follows.

* "Mixture B" consisted of three parts dried grains, three parts maize gluten feed, one part crushed oats, one part crushed wheat and one part soya cake.

The fatty acids from about 500 g. of butter-fat are distilled in a current of steam for about 4 or 5 hours and the acids volatile in steam are extracted from the aqueous condensate by ether and fractionated as such, mainly at atmospheric pressure; the extracted aqueous liquors and the recovered distilled ether are titrated with alkali, any acid present being calculated as butyric acid. The acids non-volatile in steam are recovered and weighed and a suitable portion is submitted to the lead salt separation, the resulting groups of acids being converted into methyl esters and fractionated quantitatively in the usual way.

In connection with the separation of the volatile acids, emphasis may be laid on the necessity, in the preliminary saponification of the butter-fat, for employing alcohol which is entirely free from traces of esters; it is advisable to boil the alcohol with excess of potassium hydroxide prior to use in the hydrolysis of the butter-fats, and subsequently to distil it from the potash.

It has also been observed that the residue left after fractionating the extracted volatile acids almost invariably contains a very small amount of unsaturated fatty acid, but no unsaponifiable matter. A correction for the unsaturated material present has been made on the assumption that this is oleic acid; the quantity of unsaturated acid present in the steam-distillate is so small that we have not been able definitely to identify it, but on the other hand, we have established that distillation of oleic acid in steam, under the conditions of time and rate of distillation used in these analyses, results in small amounts of oleic acid being found in the condensate in amount of the same order as those found in the butter analyses.

The composition of the acids non-volatile in steam was determined in exactly the same manner as that described in the former communication [cf. Hilditch and Jones, 1929, pp. 80, 81]: each group of neutral methyl esters (mainly saturated and mainly unsaturated) was fractionally distilled from a Willstätter bulb under high vacuum and the first 25–30 % of the primary distillate was collected in a single fraction, whilst the remainder was collected in relatively small fractions; the first primary fractions were then redistilled and collected in small successive fractions, especially in the earlier stages. Owing to the low boiling-point of methyl caprylate and caprate, it is advisable to distil the lower boiling fractions somewhat slowly and to cool the side-arm and Perkin receiver by means of filter-paper moistened with ice-cold water.

During the refractionation of the lowest-boiling primary fraction of the esters of the "solid" acids, and also (although less prominently) of the esters of the "liquid" acids from butter-fats, slight maxima have almost invariably been observed in the iodine values of fractions which consist mainly of methyl caprate and laurate. Possibly this is due to minute amounts of unsaturated esters of corresponding molecular weight, but more probably to increase in the concentration of methyl oleate in the distillate at these points; that methyl oleate readily forms constant boiling mixtures with saturated esters of a wide range of molecular weight is well known [cf. Crowther and Hynd, 1917], and

it may be that the tendency to form such mixtures is greater in the case of specific members of the saturated series.

The method of calculation has also been described at length in the earlier paper and it is perhaps only necessary here to tabulate the final results obtained in the case of each butter-fat; to illustrate the details of the procedure, however, the whole of the experimental data is quoted at length for the analysis of the component fatty acids of butter-fat IV.

Fatty acids of butter-fat IV.

Table II. *Acids volatile in steam.*

No.	g.	B.P. (° C.)	Pressure	Mean equivalent	Acids: Butyric	Caproic	Caprylic	Capric	Oleic
1	In aqueous solution (3750 cc.)			—	0·50				
2	In recovered ether (2760 cc.)			—	0·08				
3	68·41	35–100	Atmospheric	—	3·19				
4	1·64	100–160	,,	—	1·17				
5	2·47	160–165	,,	91·3	2·10	0·37			
6	3·91	165–170	,,	93·3	2·99	0·92			
7	2·04	170–175	,,	94·9	1·43	0·61			
8	2·41	175–177	,,	97·3	1·46	0·95			
9	1·93	93–120	Reduced	105·6	0·60	1·33			
10	1·29	120–132	,,	120·6	—	1·04	0·25		
11	0·85	132–135	,,	133·3	—	0·28	0·57		
12	0·82	Residue (iodine value 14·4)		176·9	—	—	0·14	0·55	0·13
					13·52	5·50	0·96	0·55	0·13

Table III. *Acids non-volatile in steam*, 394 g.

(Sap. equiv. 254·0; iod. val. 45·8.)

Lead salt separation.

	g.	%	Corresponding esters: S.E.	I.V.
'Solid' acids S	173·2	57·8	269·8	19·45
'Liquid' acids L	126·2	42·2	269·0	77·14

Table IV. *Fractionation of methyl esters.*

(i) *Esters of 'solid' acids S.*

Primary fractions: No.	g.	B.P./1 mm.	Sap. eq.	I.V.	Refractionations: No.	g.	B.P./1 mm.	Sap. eq.	I.V
					S 11	0·86	64–87°	178·8	8·0
					S 12	1·27	87–102	205·5	5·9
					S 13	3·09	102–110	234·5	3·4
S 1	43·41	66–127°	249·7	6·0	S 14	10·73	110–117	245·0	3·1
					S 15	4·14	117–126	254·0	3·45
					S 16	12·43	126–130	262·6	5·3
					S 17	6·24	Residue	279·9	16·85
S 2	11·95	127–128	265·5	9·8					
S 3	18·68	128	269·9	12·55					
S 4	7·62	128–133	271·2	14·55					
S 5	9·28	133–135	276·9	17·1					
S 6	21·87	135–137	277·4	23·1					
S 7	8·47	137–143	286·5	31·2					
S 8	21·62	143–156	288·5	37·7					
S 9	20·84	Residue	292·0	37·8					

Table IV (*contd.*).

(ii) *Esters of 'liquid' acids L.*

Primary fractions					Refractionations				
No.	g.	B.P./1 mm.	Sap. eq.	I.V.	No.	g.	B.P./1 mm.	Sap. eq.	I.V.
					L 11	1·27	40–49°	163·9	2·6
					L 12	2·87	49–54	181·9	10·5
					L 13	2·24	54–64	192·9	11·2
L 1	33·18	30–132°	225·4	35·2	L 14	1·36	64–75	207·1	10·5
					L 15	1·50	75–85	218·3	11·9
					L 16	6·25	85–99	227·6	18·85
					L 17	12·45	Residue	274·3	66·85
L 2	7·02	132–137	278·5	85·1					
L 3	12·13	137–140	286·3	85·4					
L 4	12·19	140–142	290·6	93·45					
L 5	21·10	142–143	291·3	95·8					
L 6	20·61	143–148	297·0	96·85					
L 7	11·77	Residue	315·0	114·0					

Table V. *Estimated composition of fatty acids.*

Methyl esters of 'solid' acids S.

	S 11 g.	S 12 g.	S 13 g.	S 14 g.	S 15 g.	S 16 g.	S 17 g.	Total g.	% as esters
Caprylate	0·36	—	—	—	—	—	—	0·36	0·9
Caprate	0·42	0·52	—	—	—	—	—	0·94	2·4
Laurate	—	0·66	0·91	—	—	—	—	1·57	4·1
Myristate	—	—	2·06	9·77	2·39	3·60	—	17·82	46·0
Palmitate	—	—	—	0·57	1·58	8·07	3·87	14·09	36·4
Stearate	—	—	—	—	—	—	1·14	1·14	2·9
Oleate	0·08	0·09	0·12	0·39	0·17	0·76	1·23	2·84	7·3

	S 1 g.	S 2 g.	S 3 g.	S 4 g.	S 5 g.	S 6 g.	S 7 g.	S 8 g.	S 9 g.	Total g.	% as esters	% as fatty acids
Caprylate	0·40	—	—	—	—	—	—	—	—	0·40	0·2	0·2
Caprate	1·05	—	—	—	—	—	—	—	—	1·05	0·6	0·6
Laurate	1·76	—	—	—	—	—	—	—	—	1·76	1·1	1·1
Myristate	19·96	2·90	2·13	0·66	—	—	—	—	—	25·65	15·7	15·6
Palmitate	15·78	7·68	13·82	5·67	6·70	15·18	3·05	6·36	3·46	77·70	47·5	47·5
Stearate	1·28	—	—	—	0·73	0·81	2·34	5·75	8·19	19·10	11·7	11·7
Oleate	3·18	1·37	2·73	1·29	1·85	5·88	3·08	9·51	9·19	38·08	23·2	23·3

Methyl esters of 'liquid' acids L.

	L 11 g.	L 12 g.	L 13 g.	L 14 g.	L 15 g.	L 16 g.	L 17 g.	Total g.	% as esters
Caprylate	1·01	1·02	0·09	—	—	—	—	2·12	7·6
Caprate	0·23	1·54	1·89	0·58	0·11	—	—	4·35	15·6
Laurate	—	—	—	0·63	1·21	4·77	0·76	7·37	26·4
Myristate	—	—	—	—	—	0·26	3·10	3·36	12·0
C_{18} unsat.	0·03	0·31	0·26	0·15	0·18	1·22	8·59	10·74	38·4

	L 1 g.	L 2 g.	L 3 g.	L 4 g.	L 5 g.	L 6 g.	L 7 g.	Total g.	% as esters	% as fatty acids
Caprylate	2·52	—	—	—	—	—	—	2·52	2·1	2·1
Caprate	5·16	—	—	—	—	—	—	5·16	4·4	4·25
Laurate	8·75	0·85	0·54	—	—	—	—	10·14	8·6	8·45
Myristate	3·99	—	0·90	0·43	0·22	—	—	5·54	4·7	4·7
Oleate }	12·76	6·17	10·69	11·76	20·88	20·61	11·07	82·01	69·5	69·8
Linoleate }								11·93	10·1	10·1
Unsaponifiable	—	—	—	—	—	—	0·70	0·70	0·6	0·6

Table VI. *Summarised experimental data for fatty acids of butters I–V.*

Acid	Volatile acids g.	Acids non-volatile in steam: Solid acids S g.	Acids non-volatile in steam: Liquid acids L g.	Total	% (excluding unsaponifiable matter)
		Butter-fat I.			
	9·46	81·17	79·83	170·46	—
Butyric	5·27	—	—	5·27	3·1
Caproic	2·92	—	—	2·92	1·7
Caprylic	1·27	—	1·45	2·72	1·6
Capric	—	—	3·55	3·55	2·1
Lauric	—	0·08	5·75	5·83	3·4
Myristic	—	8·25	3·45	11·70	6·9
Palmitic	—	49·29	—	49·29	29·0
Stearic	—	12·90	—	12·90	7·6
Arachidic	—	1·54	—	1·54	0·9
Oleic	—	9·04	59·06	68·10	40·1
Linoleic	—	—	6·21	6·21	3·6
Unsaponifiable	—	0·07	0·36	0·43	—
		Butter-fat II.			
	34·48	298·85	232·15	565·48	—
Butyric	19·43	—	—	19·43	3·4
Caproic	10·97	—	—	10·97	2·0
Caprylic	1·94	—	4·18	6·12	1·1
Capric	1·62	2·39	13·93	17·94	3·2
Lauric	0·24	15·54	25·08	40·86	7·3
Myristic	—	79·50	16·72	96·22	17·1
Palmitic	—	141·94	9·75	151·69	27·0
Stearic	—	27·20	—	27·20	4·8
Oleic	0·28	31·68	146·47	178·43	31·7
Linoleic	—	—	13·23	13·23	2·4
Unsaponifiable	—	0·60	2·79	3·39	—
		Butter-fat III.			
	32·74	288·0	242·0	562·74	—
Butyric	20·35	—	—	20·35	3·6
Caproic	8·42	—	—	8·42	1·5
Caprylic	1·77	0·29	7·50	9·56	1·7
Capric	1·82	2·02	17·42	21·26	3·8
Lauric	—	4·61	31·94	36·55	6·5
Myristic	—	54·44	4·84	59·28	10·6
Palmitic	—	147·16	—	147·16	26·3
Stearic	—	46·37	—	46·37	8·3
Arachidic	—	6·91	—	6·91	1·2
Oleic	0·38	26·20	157·57	184·15	32·9
Linoleic	—	—	20·33	20·33	3·6
Unsaponifiable	—	—	2·40	2·40	—
		Butter-fat IV.			
	20·66	227·9	166·1	414·66	—
Butyric	13·52	—	—	13·52	3·3
Caproic	5·50	—	—	5·50	1·3
Caprylic	0·96	0·53	3·41	4·90	1·2
Capric	0·55	1·42	7·08	9·05	2·2
Lauric	—	2·41	14·06	16·47	4·0
Myristic	—	35·47	7·74	43·21	10·4
Palmitic	—	108·10	—	108·10	26·1
Stearic	—	26·72	—	26·72	6·5
Oleic	0·13	53·25	115·91	169·29	40·9
Linoleic	—	—	16·86	16·86	4·1
Unsaponifiable	—	—	1·04	1·04	—

Table VI (*contd.*).

Acid	Volatile acids g.	Acids non-volatile in steam: Solid acids S g.	Acids non-volatile in steam: Liquid acids L g.	Total	% (excluding unsaponifiable matter)
		Butter-fat V.			
	27·24	246·3	196·7	470·24	—
Butyric	16·30	—	—	16·30	3·5
Caproic	7·89	—	—	7·89	1·7
Caprylic	1·14	—	5·10	6·24	1·3
Capric	1·59	—	12·83	14·42	3·1
Lauric	—	4·15	14·90	19·05	4·1
Myristic	—	29·48	22·77	52·25	11·1
Palmitic	—	127·80	—	127·80	27·3
Stearic	—	53·85	—	53·85	11·5
Arachidic	—	3·01	—	3·01	0·6
Oleic	0·32	27·78	118·80	146·90	31·3
Linoleic	—	—	20·95	20·95	4·5
Unsaponifiable	—	0·23	1·35	1·58	—

Discussion.

The experimental data for the four butter-fats from the Shinfield herd and those for the New Zealand sample V may usefully be considered in conjunction with the results obtained formerly from market samples of New Zealand butter.

Table VII *a*. *Summary of fatty acid analyses (weight percentages) of butter-fats.*

Butters from Shinfield, Reading.

Acid	I (Autumn-fed, 1928) %	II (Coconut cake, 1929) %	III (Soya cake, 1929) %	IV (Early summer pasture, 1929) %
Butyric	3·1	3·4	3·6	3·3
Caproic	1·7	2·0	1·5	1·3
Caprylic	1·6	1·1	1·7	1·2
Capric	2·1	3·2	3·8	2·2
Lauric	3·4	7·3	6·5	4·0
Myristic	6·9	17·1	10·6	10·4
Palmitic	29·0	27·0	26·3	26·1
Stearic	7·6	4·8	8·3	6·5
Arachidic	0·9	—	1·2	—
Oleic	40·1	31·7	32·9	40·9
Linoleic	3·6	2·4	3·6	4·1

New Zealand butters.

	(Pasture-fed, December, 1928)	Market samples [Hilditch and Jones, 1929]		
	V %	"A" %	"B" %	"C" %
Butyric	3·5	3·4	3·1	3·2
Caproic	1·7	1·8	1·9	1·7
Caprylic	1·3	0·9	0·8	0·8
Capric	3·1	1·9	2·0	2·3
Lauric	4·1	3·1	3·9	4·3
Myristic	11·1	9·7	10·6	10·8
Palmitic	27·3	27·6	28·1	28·4
Stearic	11·5	12·2	8·5	9·4
Arachidic	0·6	0·7	1·0	0·5
Oleic	31·3	34·3	36·4	33·1
Linoleic	4·5	4·4	3·7	5·4

Table VII *b*. *Summary of fatty acid analyses (molar percentages) of butter-fats.*

Butters from Shinfield, Reading.

Acid	I (Autumn-fed, 1928) %	II (Coconut cake, 1929) %	III (Soya cake, 1929) %	IV (Early summer pasture, 1929) %
Butyric	8·4	9·0	9·6	8·9
Caproic	3·5	3·9	3·0	2·7
Caprylic	2·7	1·7	2·8	2·0
Capric	2·9	4·3	5·1	3·0
Lauric	4·1	8·3	7·5	4·7
Myristic	7·2	17·2	10·7	10·9
Palmitic	27·1	24·1	23·7	24·3
Stearic	6·4	3·9	6·7	5·4
Arachidic	0·7	—	0·9	—
Oleic	33·9	25·7	27·0	34·6
Linoleic	3·1	1·9	3·0	3·5

New Zealand butters.

	(Pasture-fed, December, 1928)	Market samples [Hilditch and Jones, 1929]		
	V %	"A" %	"B" %	"C" %
Butyric	9·2	9·2	8·4	8·7
Caproic	3·4	3·7	3·9	3·4
Caprylic	2·2	1·4	1·3	1·4
Capric	4·2	2·7	2·8	3·1
Lauric	4·7	3·7	4·6	5·1
Myristic	11·5	10·2	11·0	11·2
Palmitic	25·0	25·7	26·2	26·3
Stearic	9·5	10·2	7·1	7·8
Arachidic	0·5	0·5	0·8	0·6
Oleic	26·1	28·9	30·8	27·9
Linoleic	3·7	3·8	3·1	4·5

The percentage compositions of the fatty acids in these eight samples are collected in Tables VII *a* and VII *b*; in Table VII *a* will be found the percentages by weight as determined in the analyses, whilst, since a much clearer view of some aspects of the variations in composition is obtained by considering the relative numbers of molecules of the different acids present, the corresponding molar percentages for the eight samples are given in Table VII *b*.

If the Shinfield butters are first of all considered separately, either as regards their component fatty acids (Table VII) or their general analytical characteristics (Table I), it will be seen that sample I (obtained in late autumn, 1928, with the herd on diet consisting mainly of normal winter rations with some grass) resembles butter-fat IV (from the cows on pasture in early summer, 1929) much more closely than do samples II and III (in which special fats were included in the diet). The most noticeable difference between fats II and III and fats I and IV is a quite marked diminution in the proportion of unsaturated acids present. It had originally been hoped that the autumn-fed 1928 butter (I) might serve as a starting-point for comparison of the effects of added fat in the diet, but whilst conceivably a coconut cake diet might lower the proportion of unsaturated acids, such a result would not be antici-

pated in the case of a diet including soya cake. Moreover, these data are quite at variance with the general results (as measured by the iodine values of the butter-fats) obtained in a similar systematic examination of the influence of fatty diet on milk-fat made by Holland and Buckley [1918, 1923].

These workers employed a herd of four cows (comparatively fresh in lactation) and maintained two of the cows on a daily ration of 24 lb. of hay and 10 lb. of a grain mixture throughout; the diet of the other two cows was augmented for about a month at a time by $\frac{3}{4}$ lb. daily of different fatty oils, coconut, ground-nut, maize and soya bean oil being administered during successive periods. The iodine values of butter-fats obtained from the individual cows, and the dates on which the milk samples were taken, were as follows:

	Cow no.	1	2		3	4
		Normal rations only			Corresponding period with oils	
	Daily ration					
Nov. 18–20, 27–29, 1919	24 lb. hay + 10 lb. grain mixture	27·3	28·0	No oils	27·7	27·6
Dec. 11–13, 25–27, 1919	24 lb. hay + $\frac{3}{4}$ lb. coconut fat	28·8	27·3	C.N.O.	25·3	26·5
Jan. 8–10, 22–24, 1920	24 lb. hay + $\frac{3}{4}$ lb. ground-nut oil	27·6	27·7	G.N.O.	36·2	38·0
Feb. 5–7, 19–21, 1920	24 lb. hay + $\frac{3}{4}$ lb. maize oil	27·9	28·3	M.O.	39·9	40·4
Mar. 4–6, 18–20, 1920	24 lb. hay + $\frac{3}{4}$ lb. soya bean oil	28·3	28·6	S.B.O.	41·1	41·2

Holland and Buckley state that whilst coconut oil decreased the content of butyric-capric acids and also of oleic acid and increased the proportion of lauric and myristic acids, the other three oils caused an increase in the amount of butyric, stearic and oleic acids and a progressively increasing fall in the proportions of the acids from caproic to palmitic; and they conclude that feeding with different oils and fats definitely modifies the chemical composition of butter-fat.

It is further noticeable in Holland and Buckley's experiments (i) that the cows fed only on normal rations yielded butter-fat of approximately the same (and abnormally low) iodine value all the time, and (ii) that the daily amount of additional fat given was of the same order as that present in the coconut cake and soya bean cake feed of the cows for our samples II and III. In the experience of these workers, coconut oil in the diet caused a slight but definite fall in the iodine value of the butter-fat and the presence of the other three oils in the diet caused marked increases in the corresponding iodine values, these increases being moreover roughly proportionate to the iodine values of the oils administered.

It seemed probable, on the contrary, that our own results were influenced by some condition or conditions producing a greater effect than the mere presence of specific fats in the diet, and, since the same group of ten Shorthorn cows was to be kept during the winter of 1929–1930 on the same normal winter ration (without added oil cake) as during the previous winter, it was decided

to conduct a brief examination of the fat from milk collected at approximately monthly intervals during this winter season and after the cows had returned to pasture in the spring of 1930. The results of this further study are collected in Table VIII.

Table VIII. *Butter-fats from herd of ten Shorthorn cows, Shinfield, November*, 1929–*June*, 1930.

Milk collected	Conditions	Iod. value	Sap. eq.	R.M. value	Polenske value	Kirschner value
Nov. 20–21, 1929	Indoor winter feed	42·2	248·1	27·0	1·8	21·3
Dec. 18–19, 1929	„	38·3	247·6	29·3	1·7	22·6
Feb. 4–5, 1930	„	38·9	247·9	27·3	1·6	21·4
Feb. 25–26, 1930	„	36·5	244·6	29·7	2·0	23·8
Mar. 16–17, 1930	„	37·3	246·8	30·6	1·8	24·8
Apr. 26–27, 1930	Spring pasture	43·3	244·6	28·4	1·9	23·5
June 2–3, 1930	„	44·2	249·5	24·6	1·8	20·6

The iodine values in Table VIII show that the November, 1929 sample was of practically the same degree of unsaturation as butter-fat I (end of October, 1928), and that successive samples showed a drop in iodine value which was at a maximum at about the end of February, 1930; on returning to spring pasture the iodine value was at once restored to a figure somewhat in excess of the November sample, the parallelism with the 1928–1929 observations being complete in this respect, and the restoration of the iodine value after return to pasture being extremely well defined.

It is reasonable, in view of these figures, to infer that a similar progressive variation was in progress during the 1928–1929 season and, in the light of Holland and Buckley's observation that added coconut oil in the diet does not seriously diminish the iodine value of the fat, it may also be concluded that the iodine value of butter-fat II (31·6) is not greatly below the minimum iodine value of butter-fat from this group of cows fed on the normal ration at the corresponding period of the 1928–1929 winter. If this be the case it follows that the observed content of oleic and linoleic acids in the soya cake-fed butter III represents a slight increase over the normal figure.

It is also apparent that the decrease in iodine value during the 1928–1929 season was considerably greater than the corresponding decrease in 1929–1930. Further, the minimum iodine value of the 1929–1930 series was observed towards the end of February, 1930, and butter-fats II and III were prepared from milk collected about the middle of March, 1929; thus the minimum iodine value observed in the 1929–1930 series coincided with the coolest period of a particularly mild winter, whilst samples II and III of the 1928–1929 season were obtained immediately following the intense cold of February, 1929. These facts, particularly the exceptionally low iodine values of the March, 1929 butters, suggest that an important factor in the composition of butter-fat may be the atmospheric temperature, at all events during the winter months; we believe that the results now presented indicate a general change in the composition of the butter-fat, defined more particularly by alteration in the

content of oleic acid, and due mainly to "winter conditions" (that is, either the change from outdoor to indoor conditions or from grass to indoor rations or both) and probably also to seasonal changes of temperature. In the case of butter-fats I–IV there is little doubt that causes of this description have exerted far more influence on the composition of the fat than any addition of fatty oil to the diet.

We may add that the appearance of a more saturated fatty acid mixture in butter under cold conditions is, of course, in harmony with accepted views, in so far that the body reserves of fat may be more drawn upon under cold conditions, and presumably the more unsaturated portions will be more readily consumed.

The fact that, on a similar diet, the Shinfield cows exhibited change in character of the butter-fat during the winter season, whilst the cows observed by Holland and Buckley yielded, over a similar period, milk-fats of almost identical (but extremely low) iodine value, is in our opinion a further argument in support of the contention that the chemical composition of a butter-fat is mainly determined by a variety of factors, the cumulative effect of which is certainly much greater than that produced by direct ingestion of fatty oils.

The consistently low iodine values of the butter-fats from the cows fed on the "normal" ration, in the experiments of Holland and Buckley, may be connected with the fact that the cows in question were comparatively early in lactation. Other figures given by these authors go to show that the amount of unsaturated acids in butter increases with the time that the cows have been in lactation. The Shinfield herd, from which our samples of butter were derived, was made up of cows which were at an intermediate or comparatively late stage in lactation.

It may also be pointed out that the "normal" ration given by Holland and Buckley consisted of dried hay and grain mixture, whereas that for the Shinfield cows included a substantial proportion of "roots"[1] with some hay and grain mixture; this difference in the basal diet may further have affected the composition of the resulting butters.

In this connection, and further to illustrate the difficulty of distinguishing between the various determining factors, it is worthy of note that the total amount of unsaturated components in Holland and Buckley's butter-fat from cows fed on soya bean cake, expressed in terms of iodine value (41·1), is no greater than that which we found for the "normal" butter-fats I (winter-fed, iodine value 41·3) and IV (spring pasture, iodine value 41·6) or for the corresponding fats obtained in the following season, namely, November, 1929 (iodine value 42·2) and April or May, 1930 (iodine values 43·3 and 44·2).

[1] It is worth recording that the butters from cows receiving cabbage (35 lb.) or kale (50 lb.) with 10 lb. hay (October 1928 and November 1929) had respective iodine values 41·3 and 42·2, and that those produced from December 1929—March 1930 (iodine values 36·5–38·9) were from a diet of mangolds (40 lb.) and hay (14 lb.).

In the next place, attention may be drawn to the corresponding details for the New Zealand butters. All of these possess lower iodine values than the pasture-fed English butter IV and, notably, the New Zealand December pasture-fed butter-fat V (which may be supposed, *ceteris paribus*, to be a close analogue of butter-fat IV) has a much smaller proportion of unsaturated acids and, in fact, is curiously analogous in composition to the English winter butter-fat III. Although the total amount of unsaturated acids is thus lower in the New Zealand butters studied than in butters I–IV, the proportion of linoleic acid in the unsaturated acids is consistently higher in the New Zealand samples (10–14 % of the total unsaturated acids) than in the four Shinfield butters (7–10 %). Apart from this and from the not unusual variation in stearic acid contents, the experimental values for all four New Zealand butters are of much the same order, the differences in some cases being almost within the error of experiment. The point to which we desire to draw attention is the general difference (especially in the unsaturated acid content)[1] between pasture-fed New Zealand and Shinfield butters, as disclosed in these analyses, and emphasis is laid on the fact that such differences may be as great as those observed under the most extreme variation in feeding and other conditions of the same herd throughout an annual cycle. Our results, indeed, emphasise the complexity of a problem such as the present, in which the composition of a milk-fat probably depends on indoor or outdoor life, climate, feeding and similar conditions and, possibly above all, on the individual reaction of different animals to any or all of these influences.

On the other hand, although we have felt it necessary to stress the foregoing aspects of the experimental evidence, several points of detail show quite clearly the definite though minor influence of the fatty oils which were administered to the cows in the case of butters II and III.

The chief variations in molar percentages of fatty acids (apart from butter-fats II and III) are observed in myristic and stearic acids on the one hand and in oleic acid on the other, the maximum difference even in the latter case, however, not exceeding 9 mols. %. In the whole series of eight butters, it will be noticed that within broad limits the proportion of palmitic acid does not vary very widely, the extreme values in molar percentages being 23·7 to 27·1; it was mentioned in the former paper [Hilditch and Jones, 1929, p. 86] that this proportion is, moreover, not far removed from that which obtains in most beef tallows. Again, as was also suggested in the previous paper, butyric and myristic acids are present in something approaching equimolecular proportions in all cases, with the exception of butter-fat II (where the diet had included coconut cake).

[1] If the suggestion is correct that low iodine values of the Shinfield butter-fats may be correlated with low temperature conditions, there may perhaps be a similar connection between the somewhat greater variation in day and night temperatures in New Zealand than in England on the one hand and the relatively lower unsaturation of the New Zealand pasture-fed butters on the other.

The points which appear to be of outstanding interest in the butter-fats II and III (from cows fed on an oil cake diet) are as follows.

(1) In the case of coconut cake feed the amount of lauric acid is definitely increased, but in proportionately less degree than the increase in myristic acid content; the latter rises from about 7 to 17 mols. %, whereas the lauric acid content is only increased from 4 to 8 mols. %. This is somewhat surprising in view of the fact that coconut oil contains nearly 50 % of lauric acid and only about 17 % of myristic acid; it is perhaps an indication that lauric acid is more readily transformed to other products during digestive processes than is myristic acid, and the same may be said of caprylic and capric acids, both of which occur in fair quantity in coconut oil, whilst the butter-fat II does not contain an abnormal amount of either of these components.

(2) In the case of soya bean cake feed the detailed composition is even more interesting because, although the oleic acid content is slightly above that of the corresponding coconut cake-fed butter, the linoleic acid figure is of the usual relative order, or at all events the proportion of linoleic to oleic acid is not increased by more than about 10 %. In soya bean oil, on the other hand, the relative proportions of linoleic and oleic acids are something like 2 to 1, so that practically none of the extra linoleic acid ingested by the animal has appeared unchanged in the milk. The only other marked difference between the soya cake-fed fat and either of the corresponding more normal butters I and IV (other, of course, than the general drop in unsaturation which we ascribe to extraneous conditions) is the presence of a definitely larger proportion of lauric acid than usual, which, curiously enough, approaches that found in the butter from coconut cake-fed cattle, although there is, of course, no lauric acid present in soya bean oil.

The results of Holland and Buckley have been compared with the present data in detail since the experimental material is of the same type in each case and the investigators in question carried out an exceptionally large number of systematic feeding and control tests. Reference should also be made, however, to the work of Cranfield [1916], who examined the butter-fats from cows fed during the spring of 1916 on a ration of 56 lb. mangolds, 19 lb. hay, 1 lb. of bran and ½ lb. of dried yeast, with 4 lb. of either decorticated ground-nut or decorticated cotton seed cake; the resulting butter-fats were in each case of much the same composition (Reichert-Meissl values 29·3–32·3, Polenske values 1·87–1·96, Kirschner values 22·0–24·2, iodine values 38·4–43·8), and it will be noticed that these values are of the same general types as those for the Shinfield autumn butters of 1928 and 1929. Cranfield considered that the amount of unsaturated acids was rather greater, on the whole, after ground-nut cake than after cotton-seed cake diet; ground-nut oil fatty acids contain about 55–65 % oleic and 20–25 % linoleic acid, whereas in cotton-seed oil fatty acids the respective proportions are about 25 % oleic and 45–50 % linoleic acid.

An observation by Iyer [1919], to the effect that rice polish used with a ration of linseed cake for cows has the result of increasing the volatile acids

and diminishing the unsaturated acids in the butter-fat (ghee), may have some bearing on the influence of bran and grain fed to cattle in "grain mixtures," and interesting results might be obtained by investigating the influence of other components of the diet than oil-cake on the fatty acid composition of butters.

The comprehensive paper by Channon, Drummond and Golding [1924] on the effect of certain oils in the diet of cows upon various characteristics of butter-fat has special reference to cod-liver oil, but the effects of ground nut and coconut oils were also studied with results very similar to those observed by us in the respective cases of soya bean and coconut oils. Moreover, the iodine values of the butters throughout the winter and spring seasons of 1922–1923 at Shinfield show the same general trend of variations as in the present work.

SUMMARY.

Detailed analyses have been made of the fatty acids present in four English butter-fats obtained from members of the same herd of cows fed on various diets, and in a New Zealand pasture-fed (December) butter-fat, with the object of tracing any effects due to the ingestion of fatty oil. The results show that the influence of added fat in the diet, although definite, is of a minor order compared with that due to other causes.

Much more profound changes in fatty acid composition appear to be caused by conditions such as the change from outdoor to indoor life, the general character of the diet (*e.g.* pasture, "roots," hay, etc.), and seasonal changes in the temperature. Additional information on the incidence of these factors was obtained from a survey of the characteristics of butters obtained monthly from the same herd of cows when receiving a standard diet (without oil-cake).

The greater differences which are believed to be associated with such factors as the type of diet, the indoor or outdoor life, or the seasonal temperature, lie mainly in variation of the amount of unsaturated acids present, and in the amount of the lower saturated acids (butyric to lauric); palmitic acid appears relatively to be the most constant in proportion of any of the components (24–27 %), whilst the stearic acid content varies somewhat erratically from one butter to another.

Taken as a whole, all the butter-fats which have been analysed in detail in this and in a preceding investigation [Hilditch and Jones, 1929] are characterised by amounts of butyric to myristic acids which are more or less specific for cow milk-fat, and by amounts of palmitic, oleic and linoleic acids which, although equally characteristic of most butter-fats, are also not widely dissimilar from the proportions of the same acids which are found in the body- or storage-fats of the same class of animal.

We desire to express our thanks to Captain Golding and the National Institute for Research in Dairying for their considerate assistance in this work,

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CXXVI. THE COLORIMETRIC DETERMINATION OF CYSTINE BY MEANS OF THE URIC ACID REAGENT.

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The colorimetric method for the determination of cystine, originally proposed by Folin and Looney [1922], is based upon the fact that cystine in the presence of sodium sulphite is reduced to cysteine, which reacts in alkaline solution with the phosphotungstic acid reagent of Folin and Denis [1912], termed by them the "uric acid reagent," giving rise to a blue colour.

The original procedure has been modified from time to time. Folin and Trimble [1924] sought to make the reagent more specific, in particular to eliminate the sensitivity towards tyrosine and other phenols, by employing a reagent from which molybdenum compounds had been substantially removed. In a more recent investigation, Folin and Marenzi [1929, 1] have discovered another defect from which the original method suffered, namely, that in order to obtain complete reaction with the cystine relatively much more of the uric acid reagent has to be added than was employed by Folin and Looney.

In this paper Folin and Marenzi also recommend adding the sulphite (to reduce the cystine to cysteine) before and not after the alkali is added, and they use a reagent from which all traces of molybdate have been removed [1929, 2].

Whilst former procedures have given only a limited degree of proportionality between cystine content and colour intensity, that now described by Folin and Marenzi shows strict proportionality over a range at least from 1 to 4 mg. of cystine.

In attempting to use this latest reagent and procedure, the writer encountered two difficulties.

(1) Turbidity very frequently formed in both standard and test solutions.

(2) Small inaccuracies in measuring out the saturated sodium carbonate solution appeared to produce quite appreciable changes in colour intensity.

Since the procedure appears in all other respects to be eminently suitable for quantitative colorimetric work, it was decided to make studies in the following directions.

(1) The effect upon the colour intensity of variations in the quantity of

sodium carbonate added. In view of the marked influence of the latter, "saturated" sodium carbonate as recommended by Folin and Marenzi does not define the concentration with sufficient precision.

2. The use of sodium hydroxide, as recommended by Hunter and Eagles [1927] in place of sodium carbonate, the use of which they criticise on the grounds that on the addition of the acid reagent, carbon dioxide is liberated, which in turn liberates sulphurous acid from the sulphite thereby causing quite a large blank owing to the reduction of the phosphotungstic acid.

(3) The conditions giving maximum colour intensity with the minimum of interference from turbidity. Having had little success himself in the search for a substance inhibiting turbidity, the author wrote to Prof. Folin who drew attention to his use of urea to achieve this object, when determining uric acid, in a paper just then published [Folin, 1930].

The author is indebted to Prof. Folin for his suggestion, which, as will appear in the experimental part, was adopted with complete success in suppressing turbidity in the present investigation.

Experimental.

The following solutions were employed.

(1) Uric acid reagent of Folin and Marenzi.

(2) Standard cystine solution containing 1 mg. of cystine in 2 cc. of 0·1 *N* hydrochloric acid.

(3) "Saturated" sodium carbonate solution, actually 3·6 *N*, also a 3 *N* solution, both made from A.R. sodium carbonate.

(4) CO_2-free sodium hydroxide, *N* solution.

(5) Freshly prepared 20 % solution of Merck's sodium sulphite (for reduction of cystine to cysteine).

(6) Freshly prepared 3 % solution of Merck's sodium sulphite (for diluting to final volume).

(7) A 20 % solution of lithium sulphate.

(8) A 40 % solution of urea.

Tests were made using either 1 or 1·5 mg. of cystine, as stated, together with 1 cc. of 20 % sulphite, and 4 cc. of reagent, the final volume being made up to 50 cc. (unless otherwise stated). Thus, although the quantities are one half of those employed by Folin and Marenzi, the relative proportions are the same.

Influence upon colour intensity of quantity of sodium carbonate solution added.

A series of eight tests was performed, made up as shown in Table I, in which the quantity of sodium carbonate was the only factor varied. The flask to which 5 cc. of sodium carbonate had been added served as the standard against which all the others were matched. 1 cc. of lithium sulphate solution was included in each flask according to the Folin-Marenzi procedure.

Table I.

1·5 mg. cystine; final volume 50 cc.

	a	*b*	*c*	*S*	*d*	*e*	*f**	*g**
Saturated sodium carbonate cc.	1	3	4	5	6	7	8	10
Water cc.	0	7	6	5	4	3	2	0
Colour value	90·0	89·5	89·5	**100·0**	115·5	116·5	118·0	118·0

* Turbidity formed in each of these flasks whilst matching.

Folin and Marenzi recommend the use of 18 cc. of saturated sodium carbonate solution, equivalent to 9 cc. on the half-scale employed here. Although at this alkalinity the colour value tends to become constant, yet in practice it was found that turbidity invariably developed in those solutions containing 8 cc. or more of sodium carbonate. The addition of greater quantities of lithium sulphate failed to counteract this tendency.

A trial in the present instance showed that quite appreciable colour formed in a blank to which 5 cc. of saturated carbonate had been added, but a blank containing 9 cc. even after 10 minutes' standing, although turbid, remained practically without colour.

Influence upon colour intensity of quantity of N *sodium hydroxide solution added.*

These tests were carried out in the same way as those previously described, the flask selected as standard containing 5 cc. of *N* sodium hydroxide (Table II). Lithium sulphate (1 cc.) was added to each flask.

Table II.

1·5 mg. cystine; final volume 50 cc.

	a	*b*	*S*	*c*	*d*	*e**	*f**	*g**
N Sodium hydroxide cc.	1	3	5	7	8	8·5	9	10
Water cc.	0	7	5	3	2	1·5	1	0
Colour value	99·5	100·5	**100·0**	100·0	105·0	136·5	149·0	170·0

* Turbidity formed in each of these flasks.

To make 4 cc. of reagent alkaline to phenolphthalein 7·3 cc. *N* sodium hydroxide solution were required.

The general inference to be drawn from the two sets of results is that high colour intensity is not to be obtained, either with sodium carbonate or hydroxide, without accompanying liability to turbidity. In the case of the sodium hydroxide experiments, it was impossible to follow the colour value any further owing to the immediate clouding of the solutions. It is presumed that it would tend to become constant as in the case of sodium carbonate.

It is also clear that colour formation, due to the interaction of cysteine and reagent, takes place in acid solution although, since the colour developed is less than in alkaline solutions and the blank is considerably larger, this

region is not suitable for quantitative work. It will be noted that in the case of the sodium hydroxide experiments the increase in colour value occurs immediately the reaction becomes alkaline (7·3 cc. alkali).

The equivalent of sodium carbonate solution is 2 cc., however the increase did not occur until over 4 cc. had been added. Evidence obtained later on, however, showed that here also as soon as the p_H of the complete reaction mixture exceeded 7·0, greater colour intensity resulted.

Alkaline solutions are desirable in cystine determination provided turbidity can be avoided. It is not permissible to purchase stability by cutting down the alkali since, almost immediately, a range is reached where colour intensity varies directly with alkali added. In this connection it is difficult to see how Hunter and Eagles could have obtained very satisfactory results when using sodium hydroxide as alkali.

Before passing to the question of turbidity, the following experiments may be recorded which demonstrated that dilution and actual volume during colour formation do not untowardly affect final colour values. In addition, it was found that small variations in the quantity of reagent (from 4 cc. to 1·5 mg. cystine) are without effect.

(1) Tests were compared, each containing 1·5 mg. cystine, but brought to final volumes of 25 cc. and 50 cc. respectively. The weaker at 40·5 matched the stronger set at 20.

(2) Colour development was allowed to take place in similar tests, one having had 5 cc. of water added making volumes of 17 cc. and 22 cc. respectively. Diluted to 50 cc. each, the one at 21 matched the other set at 20.

The suppression of turbidity.

As stated above, it was found impossible to work with sodium carbonate in the range giving maximum colour intensity owing to the development of turbidity. Investigation showed that it was the sodium salt of the phospho-17-tungstic acid described by Wu [1920] which was being precipitated in the form of minute, glistening, rectangular platelets.

In the following experiments "saturated" sodium carbonate was replaced by a solution of exactly specified concentration. 3 *N* was selected as being suitable since this is near to saturation but sufficiently far removed to obviate the possibility of crystallisation occurring during winter months.

A mixture containing 2 cc. of cystine solution (1 mg.), 4 cc. of reagent, 5 cc. of 3 *N* sodium carbonate solution and the other reactants in their customary proportions has a p_H, measured by the hydrogen electrode, of 6·9. Inflexion of the curve relating quantity of sodium carbonate and colour intensity occurs at this point.

The experiments (see Table III) illustrating the action of urea, referred to above, in suppressing the precipitation of the interfering sodium phospho-17-tungstate, also serve to show that urea, in varying quantity, does not affect

the final colour developed, and it was speedily found that all trouble could be eliminated by using 5 cc. of a 40 % aqueous solution added to the flasks immediately prior to the reagent.

Table III.

1·0 mg. cystine; final volume 25 cc., 8 cc. of 3 *N* sodium carbonate to each; no lithium sulphate added.

	S	*a*	*b*	*c*	*d*
40 % urea solution (cc.) ...	5	4	3	2	1
Water (cc.)	0	1	2	3	4
Colour value	100·0	101·5	102·5	98·0	*
Time after mixing before turbidity develops (mins.)	(Not in 18 hours)	30	20	15	<5

* Almost immediate development of turbidity prevented any accurate comparison being made with this solution.

A complete determination should thus be carried out in the following manner.

To 2 cc. of cystine solution, or volume equivalent to about 1 or 1·5 mg. cystine, add 1 cc. of 20 % sodium sulphite (Merck's) and wait 1 minute.

Add 8 cc. of 3 *N* sodium carbonate (A.R.) solution, 5 cc. of 40 % urea solution, mix and run in 4 cc. of Folin and Marenzi's uric acid reagent.

After 5 minutes make up to 25 cc. (or 50 cc. if preferred) with 3 % sodium sulphite (Merck's) solution and proceed with the colorimetric comparison.

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CXXVII. LACTIC ACID OXIDATION IN BRAIN WITH REFERENCE TO THE "MEYERHOF CYCLE."

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In a series of papers Meyerhof [1920, 1, 2 and 3] showed that the chemical events accompanying muscular contraction revolved in a definite cycle, lactic acid being formed anaerobically from carbohydrate, and being restored aerobically, the energy for this restoration being derived from the oxidation of a portion of the lactic acid itself. Following on this work, a large number of important papers by Meyerhof, Warburg, and their collaborators, have seemed to establish the fact that the cycle was no isolated example of biological machinery, but was a general phenomenon, characteristic of most, if not of all, living cells. It will suffice here to mention three of these papers, in which the point is, perhaps, brought out most clearly [Meyerhof, 1925; Meyerhof and Lohmann, 1926; Warburg, Posener and Negelein, 1924].

In the experiments on muscle, the changes in lactic acid and the synthesis of carbohydrate were demonstrated by chemical methods. The chemical findings, moreover, confirmed, and were confirmed by, the experimental heat determinations of Hill [1922] with most striking precision. While this is true of muscle, the occurrence of a carbohydrate-lactic acid cycle in other cells has not been clearly demonstrated. The experimental work was performed, for the most part, by micro-methods, devised in the first place by Warburg, and frequently modified. They involved manometric measurements, and were performed on a scale which surely precluded accurate chemical estimation.

In the case of muscle, the ratio $\frac{\text{Total lactic acid disappearing}}{\text{Lactic acid oxidised}}$, known as the "Meyerhof quotient," has a value from 3 to 6. By the manometric method, it is possible to measure (1) the oxygen used by the tissue, (2) the CO_2 evolved by the tissue, either anaerobically or aerobically. The tissue is bathed in a solution containing bicarbonate, and kept in an atmosphere containing CO_2. Any production of strong acid, such as lactic acid, will cause the evolution of CO_2; any removal of lactic acid will cause its retention in the fluid, as bicarbonate. From the CO_2 measurements, therefore, are deduced the changes taking place in the lactic acid content of the system.

In dealing with these experiments, the authors have been accustomed to express the "Meyerhof quotient" as the ratio

$$\frac{\text{Lactic acid prevented from appearing in oxygen}}{\text{Lactic acid equivalent of oxygen used}},$$

that is, $\frac{\text{Lactic acid produced anaerobically minus lactic acid produced aerobically}}{\text{Lactic acid equivalent of oxygen used}}$.

In the case of most cells, they have obtained a "quotient" greater than unity, indicating a synthesis of carbohydrate under aerobic conditions, and therefore the existence of a "Meyerhof cycle."

The idea that this "cycle" is a fundamental principle, underlying the chemical dynamics of almost all living cells, is very attractive. At the same time, its truth, in the case of tissues other than muscle, seems to lack complete verification, since the actual demonstration of carbohydrate synthesis under experimental conditions, in which a "Meyerhof quotient" is obtainable, is lacking. With this in view, one of us (E.H.) in conjunction with Mrs B. E. Holmes, attempted, in 1926, to demonstrate the existence of a Meyerhof quotient and its relationship to carbohydrate synthesis in the case of brain tissue. Unfortunately, we were forced by circumstances to abandon the project in favour of other work. But the present experiments have fully confirmed the suggestions made by those earlier ones.

General principles of method.

It was impossible to perform the experiments on the small scale adopted by Meyerhof, Warburg and their collaborators. The following plan was therefore adopted. Two large "Barcroft" differential manometers, constructed by Flaig and Co., of Hatton Garden, London, with cups of a total capacity of 110 cc. and manometers of working length 230 mm. were calibrated to work with 5 cc. of fluid in each cup, the right hand cup containing, in addition, 2 g. of tissue. The greatest excursion of the oil corresponded to an oxygen uptake of about 1660 mm.^{3} (depending, of course, on the factor). With 2 g. of tissue, readjustment was necessary after about one hour.

These instruments were shaken in a water thermostat at 37°. Both contained chopped brain tissue, but while one lot of tissue was suspended in plain phosphate-buffered Ringer's solution, the other was suspended in Ringer's solution of the same composition and p_H, but containing in addition 0·5 % lithium lactate. The oxygen uptakes of each were measured: that of the sample in plain Ringer's solution began to fall off, usually in 60–90 minutes, while that in the lactate-Ringer's solution remained linear, or nearly so. At the end of the experiment, the difference in total oxygen uptake was measured, and represented the extra oxygen used by reason of the added lactate. At the commencement of the experiment, another two 2 g. samples of tissue had been weighed out, and immersed in 5 cc. of plain and lactate-Ringer's solution, respectively. When the taps of the manometers were first closed, alcohol was added to these "initial" samples of tissue. At the end of the experiment

the tissue was killed immediately either by the addition of alcohol or trichloroacetic acid, and lactic acid and free sugar, or lactic acid and glycogen were estimated in all four samples. For convenience, the two samples recovered from the Barcroft cups will be designated OP (no added lactate) and OL (added lactate) and, similarly, the other two, IP and IL. Then:

(IL − OL) = Total lactic acid removed from the sample of tissue to which lactate was added,

and (IP − OP) = Amount of preformed lactic acid removed from the tissue.

So that (IL − OL) − (IP − OP) = Amount of added lactic acid removed.

The oxygen consumed by OL minus the oxygen consumed by OP = extra oxygen used, and corresponds to the added lactic acid removed. In respect of this added lactic acid, therefore, the "Meyerhof quotient" is

$$\frac{\text{Added lactic acid removed}}{\text{Lactic acid equivalent of extra oxygen}}.$$

If this be greater than 1·0 the fact should, according to the "cycle" theory, indicate a synthesis of carbohydrate; and from the actual figures, the magnitude of this expected synthesis can be calculated.

Chemical methods.

In view of certain complications which recent work has brought to light in the estimation of carbohydrates, the methods employed will be described in some detail.

1. *Lactic acid and "free carbohydrate."* The term "free carbohydrate" covers carbohydrate substances soluble in 60 % alcohol, and in acid mercuric chloride (Schenk's reagent), which reduce ferricyanide under the conditions of the Hagedorn and Jensen [1923] blood-sugar method. Sufficient alcohol was added to the suspension of tissue in Ringer's solution to make the alcohol concentration 60 %. The tissue was finely ground in a mortar and washed into a 50 cc. volumetric flask. The flask was filled up to the mark with 60 % alcohol and allowed to stand for 3 hours, or longer. The contents were then centrifuged, and the volume of clear centrifugate noted (x cc.). The final result was multiplied by the factor $50/x$.

The centrifugate was evaporated to dryness in a beaker over a water-bath. The residue was rubbed up with 5 cc. of Schenk's reagent and filtered through asbestos. Three more 2 cc. portions of reagent served to wash beaker, funnel and precipitate. From the filtrate, Hg was removed by H_2S and HgS filtered off through a washed filter-paper. The precipitate was washed with water, and filtrate and washings were aerated to remove H_2S. The acid filtrate was then sometimes (see "hydrolysed values," Table II) heated for 2 hours on a boiling water-bath; whether hydrolysed or not, it was next neutralised and subjected to the "copper-lime" precipitation. The reducing substances were estimated in the precipitate, the lactic acid in the supernatant fluid. This

procedure has been previously described [Holmes and Gerard, 1929]. Two modifications only have been introduced.

(*a*) The alkalinity of the final filtrate, upon which the sugar estimations were performed, was adjusted by the method described by Kimmelstiel [1929] for the estimation of cerebroside-galactose.

(*b*) The lactic acid estimations were performed by the method of Friedemann, Cotonio and Shaffer [1927].

2. *Estimation of lactic acid and glycogen.* The difficulties introduced into the estimation of glycogen by the fact of its solubility in 60 % alcohol have already been discussed [Holmes and Gerard, 1929. See also Kerly, 1930]. To circumvent these difficulties, the following procedure was adopted. The tissue and Ringer's solution were transferred to a centrifuge tube, and washed in with absolute alcohol, in all about 30 cc. of alcohol being used. After standing for a short time, the mixture was centrifuged, and the clear fluid transferred to a beaker and evaporated to dryness on the water-bath. Meanwhile, the tissue was transferred to absolute alcohol, ground, and extracted four times, each portion of alcohol being filtered off through a Jena glass filter (mesh 5/7). When the centrifugate had evaporated to dryness, the residue (called, for convenience, residue A) was extracted 5 or 6 times with absolute alcohol, which was filtered off through the glass filter. The combined alcoholic extracts (*i.e.* that from the tissue, and that from residue A) now contained all the lactic acid, while the glycogen, being insoluble in absolute alcohol, was contained, partly in the tissue, partly in residue A. The tissue was transferred to the beaker containing residue A, and the whole was extracted four times with boiling chloroform (to remove cerebrosides) and finally washed completely, with chloroform, into the glass funnel, and there left until every trace of chloroform had evaporated. The dry, powdery, residue was then transferred to a centrifuge tube and heated for 3 hours with 0·5 cc. of 60 % KOH. It was then cooled; 0·5 cc. of water, 4 cc. of alcohol, and a few drops of bromocresol purple [Kerly, 1930] were added, and the fluid was neutralised with trichloroacetic acid. Water was then added, so that the volume of watery solution (0·5 cc. KOH + 0·5 cc. H_2O + x cc. trichloroacetic acid + y cc. bromocresol purple) was brought to 2·0 cc. The addition of a further 4·0 cc. of alcohol then gave a concentration of 80 % alcohol, 4 cc. of alcohol already being present. After standing overnight, the fluid was centrifuged and the residue washed once with 5 cc. of 90 % alcohol, and twice with 5 cc. of absolute alcohol. It was then extracted with four 3 cc. portions of boiling water, to the first of which a drop of HCl had been added. Each portion was filtered successively through the same washed filter paper into another centrifuge tube. HCl was added to make the concentration 2·2 %, and the fluid was hydrolysed for 3 hours on a boiling water-bath. If necessary, the fluid was again filtered. It was then neutralised, and the copper-lime precipitation and Hagedorn and Jensen estimation were performed as for "free carbohydrate."

The lactic acid estimation was performed as follows. The alcoholic solution

of the lactic acid was evaporated to dryness, and the residue extracted three times with 10 % trichloroacetic acid. The filtrate was treated with copper-lime, and the lactic acid estimated by the method of Friedemann, Cotonio and Shaffer [1927].

Results.

The results (Table I) of a short series of preliminary experiments, performed in each case with 0·3 g. of chopped rabbit brain, put up in 3·0 cc. of

Table I. *Disappearance of lactic acid and oxygen uptake for 0·3 g. of tissue in 3 cc. phosphate-buffered Ringer's solution at* p_H *7·6 and 37°.*

Exp.	Duration (mins.)	IL Tissue and added lactate. Initial value mg.	OL Tissue and added lactate. Final value mg.	IP Tissue alone. Initial value mg.	OP Tissue alone. Final value mg.	IL−OL mg. (A)	IP−OP mg. (B)	Added lactic disappearing (A−B)	O_2 absorbed by OL (mm.³)	O_2 absorbed by OP (mm.³)	"Extra" O_2 absorbed	Lactic equivalent of extra O_2 (mg.)	"Meyerhof quotient"	Strength of lactate employed %
1	206	7·9	6·58	0·65	0·28	1·32	0·37	0·95	1287	692	595	0·813	1·17	0·25
1*a*	206	7·72	6·70	0·70	0·35	1·02	0·35	0·67	1276	711	565	0·77	0·87	0·25
2	90	15·8	14·8	0·425	0·298	1·0	0·127	0·87	438	361	77	0·105	8·3	0·5
2*a*	180	15·8	13·5	0·425	0·143	2·3	0·283	2·02	921	505	416	0·567	3·54	0·5
3	223	5·45	4·02	0·492	0·049	1·43	0·44	0·99	1074	764	310	0·422	2·34	0·2
3*a*	223	13·8	11·50	0·492	0·049	2·3	0·44	1·86	1161	764	397	0·562	4·09	0·5
3*b*	223	17·6	15·45	0·492	0·049	2·15	0·44	1·71	1021	764	257	0·352	4·85	0·7

Exp. 1 is by the trichloroacetic extraction method.
Exp. 1*a* is by the alcohol extraction and Schenk method.
Exps. 2–3*b* are by the trichloroacetic extraction method.

Ringer's solution in Barcroft manometers of the ordinary type, show that a "Meyerhof quotient" is obtainable with 0·5 % lithium lactate. 0·25 % lactate on one occasion gave a quotient of almost 1·0, and on another, 0·2 % gave one of more than 1·0. The results obtained by working up the tissue (*a*) by the trichloroacetic acid method and (*b*) by extraction with 60 % alcohol, followed by Schenk's reagent, agree quite well. It will be noticed that the largest quotient of all (8·3) is obtained in Exp. 2, the duration of which is the shortest of the series. Exp. 2*a* was done simultaneously upon further samples of tissue from the same brain, but was allowed to run for 180 instead of for 90 minutes. Exps. 3, 3*a*, and 3*b* were also carried out simultaneously, on batches of tissue from the same animal; here the quotient rises with the concentration of lactate. The suggestion—which is being tested further—is that the shorter the duration of the experiment, and the higher the concentration of lactate, the larger the quotient is likely to be.

The results of the experiments with the large manometers, in which carbohydrates, as well as lactic acid, were estimated, are shown in Table II. In all cases, a quotient was obtained. The differences in the amounts of lactic acid estimated lie far outside the limits of experimental error, and the results are therefore put forward with confidence. The quantities of oxygen absorbed agree very well with those obtained in the preliminary experiments, if both sets of values are calculated on the basis of mm.³ of oxygen per g. of tissue

Table II. *Lactic acid, free carbohydrate, glycogen and oxygen uptake of brain tissue at p_H 7·6 and 37°. Glycogen calculated as glucose.*

	Lactic acid (mg.)							Oxygen uptake (mm.³)				
Duration of exp. (mins.)	Tissue and added lactic acid. Initial IL	Tissue and added lactic acid after shaking OL	Lactic acid disappearing IL−OL=A	Tissue alone. Initial IP	Tissue alone after shaking OP	Lactic acid disappearing IP−OP=B	Added lactic acid disappearing A−B	Tissue and added lactic acid OL	Tissue alone OP	"Extra" oxygen	Lactic equivalent of extra O_2 (mg.)	"Meyerhof quotient"
235	19·8	14·45	5·35	2·98	0·825	2·155	3·195	5770	3981	1189	1·62	1·96
276	20·8	14·35	6·45	2·53	0·41	2·12	4·33	5815	4585	1230	1·677	2·58
257	21·0	12·5	8·5	2·09	0·285	1·80	6·7	6690	4485	2205	3·01	2·23
180	22·3	16·0	6·3	2·62	0·322	2·30	4·0	4255	3550	705	0·96	4·16
240	18·8	12·7	6·1	1·60	0·20	1·40	4·7	4565	3505	1060	1·47	3·2
180	17·0	14·7	2·3	1·36	0·33	1·03	1·27	2160	1750	410	0·56	2·3
256	19·85	17·35	2·5	1·045	0·276	0·77	1·73	2720	1990	730	0·995	1·74

		Free carb. in IP and IL (mean)		Free carb. in OL		Free carb. in OP						
Duration of exp. (mins.)	Expected synthesis of carbohydrate (mg.)	unhydrolysed	hydrolysed	unhydrolysed	hydrolysed	unhydrolysed	hydrolysed	Glycogen in IP and IL (mean)	Glycogen in OL	Glycogen in OP	"Synthesis"	Wt. of tissue (g.)
235	1·57	0·712	1·26	0·925	—	0·626	1·066	—	—	—	+0·3	2·0
276	2·65	0·317	0·849	0·375	—	0·345	—	—	—	—	+0·03	2·0
257	3·7	—	0·97	—	0·660	—	0·632	—	—	—	+0·028	2·0
180	3·04	—	0·923	—	0·699	—	0·689	—	—	—	+0·01	2·0
240	3·23	—	—	—	—	—	—	1·095	0·871	0·895	−0·024	1·5
180	0·71	—	—	—	—	—	—	0·449	0·221	0·363	−0·142	1·0
256	0·74	—	—	—	—	—	—	0·466	0·279	0·361	−0·082	1·0

in 3 hours. It is unnecessary to give the complete protocols of the manometer readings; one typical curve only of the oxygen uptake is, therefore, given (Fig. 1). One point, however, must be made quite clear. The experiments

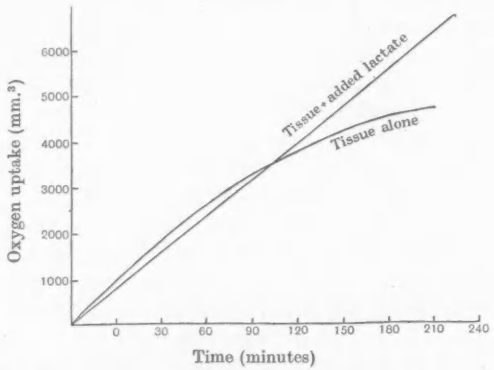

Fig. 1. Typical oxygen uptake of brain tissue, with and without added lactate. The curves are for 2 g. of tissue.

were performed at 37° and in many cases were prolonged beyond 3 hours. The possibility of bacterial infection therefore arises. The manometer cups were always kept in bichromate mixture until just before use; the constituent solutions of the Ringer's solution were always boiled just before mixing and

adjusting the p_H. (The final solutions had the following composition: mammalian Ringer's solution 80 parts, 0·2 *M* KH_2PO_4 20 parts, and were adjusted to p_H 7·4 with sodium hydroxide.) The growth of bacteria in the cup of a manometer is almost always signalised by a sharp upward bend in the O_2 absorption curves, and this never occurred in our experiments. Finally, the same results were obtained in the experiments carried on for exactly 3 hours, as in those prolonged for a greater period.

It will be seen that, in spite of the fact that a "Meyerhof quotient" was always found, there was never any synthesis of carbohydrate, either as "free carbohydrate" or as glycogen. The "free carbohydrate" fraction contained material, the reducing value of which increased on acid hydrolysis: in spite of this, the hydrolysed and unhydrolysed values were respectively identical whether the tissue had been oxygenated in the presence or absence of lactate, though both were somewhat lower than the "initial values" (*i.e.* those tissues worked up immediately). In the case of the carbohydrate, the "expected synthesis" was several times greater than the amount of carbohydrate actually found, and far beyond the limits of any possible experimental error.

In two experiments, which can only be regarded as preliminary, we have attempted to measure the respiratory quotient of the "extra" oxygen. We used the method of Keilin [1929], that is, small cups were hung on the soda pots in the manometer cups, one containing baryta and the other acid. Small Barcroft apparatus were used containing 3 cc. and 0·3 g. of tissue. In two the cups were upset immediately after closing the taps to give initial values for CO_2 in tissue and Ringer's solution and in the other two this was done after a suitable oxygen uptake had been measured. In the two experiments so far performed the R.Q.'s of the extra oxygen were 0·9 and 0·86. We place very little reliance on these two results but if confirmed they would suggest that the extra oxygen does indeed completely oxidise part of the added lactic acid.

Discussion.

It is possible that exception will be taken to these experiments on the ground that chopped brain is in an unphysiological condition, and could not be expected to behave normally. The question of the relative merits of slicing and chopping is rather a vexed one. It is quite true that finely minced muscle does not display the chemical phenomena which one associates with intact muscle—but the most striking peculiarity of minced muscle is that its power of taking up oxygen is much impaired. We find, on the other hand, that chopped brain takes up oxygen very rapidly indeed. We have found that it makes very little difference to the oxygen uptake of rabbit cortex whether it be sliced or chopped [Holmes, 1930], and Dixon and Elliott [1929] make the same claim with regard to many other tissues. We feel, therefore, that a sufficient answer to any such objection is that oxygen absorption does occur, and that a "quotient" can be demonstrated.

Warburg and Meyerhof do not seem to be agreed as to whether a quotient is actually obtained with brain tissue or not. Both authors employed slices of grey matter (rat brain). Warburg *et al.* [1924] give a single experiment in Ringer's solution in which the quotient was 1·6, while Meyerhof quotes some thirteen experiments, from which he concludes "...so dass auch hier entweder die ganze Atmung auf Oxydation der Milchsäure zu beziehen ist oder eine wenn auch geringe bilanzmässige Zunahme von Kohlehydrat stattfinden könnte."

Takane [1926] states that he estimated carbohydrates in brain; that in some of his earlier experiments there appeared to be an increase in carbohydrate, when they were conducted in oxygen in the presence of lactate, but that later experiments failed to confirm this. He gives, however, no figures. The balance of opinion seems to be that neither synthesis of carbohydrate nor the existence of a quotient can be demonstrated. We have confirmed the first of these suggestions—undoubtedly no synthesis, either of glycogen or free carbohydrate, occurs. But we have also shown that a "quotient" is most certainly obtainable.

The story must remain incomplete until we can trace the fate of that portion of the added lactic acid which disappears, over and above that which can be accounted for by the extra oxygen. Clearly there are many possibilities. For instance "synthesis" may in fact occur, but the product may be one which is not estimated by the methods we have employed. Again, it is possible that partial oxidation of the lactic acid molecule takes place, leaving a large portion of it in a condition in which it is no longer estimated as such, so that although lactic acid disappears, yet the extra oxygen is insufficient to account for its complete oxidation. Suppose for instance, that a formation of pyruvic acid took place by a removal of two atoms of hydrogen: the "quotient" would then be 6·0. This is intended merely as an illustration. It is almost certain that pyruvic acid is not formed, because (1) the very delicate sodium nitroprusside test for pyruvic acid is negative when applied to a colloidal ferric hydroxide filtrate from brain, which had been shaken with lactate in oxygen at 37° for 2 hours, (2) pyruvic acid would be estimated partially by the method for lactic acid, and (3) pyruvic acid rapidly passes to other products in the tissues. Similarly, the piperidine-nitroprusside test for acetaldehyde [Lewin, 1899] was negative when applied to the colloidal ferric hydroxide filtrate. Acetaldehyde would, on the one hand, be driven off during evaporation when the alcohol extraction method was used, and, on the other, would interfere with the lactic acid estimations when the trichloroacetic acid method was used. That substantially the same results were obtained by the two different methods is a fact of considerable importance.

We have demonstrated that a "Meyerhof quotient" is obtainable with brain tissue and added lactic acid[1], and that, in spite of this, there is not that synthesis of carbohydrate postulated by Meyerhof in his "cycle" theory. The

[1] The lithium salt used in these experiments was that of *d*-lactic acid.

fact that this is possible surely demands a suspension of judgment in the cases of those tissues where the existence of a "Meyerhof cycle" is claimed, but in which no actual synthesis has been demonstrated by chemical means.

SUMMARY.

1. Experiments are described in which lactic acid disappearance, oxygen consumption and production of carbohydrate by brain tissue, with and without added lactate, have been determined.

2. It has been shown that, although a "Meyerhof quotient" is readily obtainable under these experimental conditions, there is no synthesis of carbohydrate from that portion of the lactic acid which disappears but is not accounted for by O_2 uptake.

3. The fate of the lactic acid which disappears is not yet clear.

4. These experiments throw doubt on the reality of the alleged "Meyerhof cycle" in the case of cells in which the actual synthesis of carbohydrate has not been demonstrated by chemical estimation.

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CXXVIII. THE RATE OF LIBERATION OF ARGININE IN TRYPTIC DIGESTION[1].

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When a protein is subjected to the hydrolysing action of trypsin[2], its constituent amino-acids are liberated at different speeds and to different extents. Such differences of behaviour are the consequence, one may presume, of differences in the mode of combination, or in the order and arrangement, of the individual amino-acids within the protein molecule. Their study, therefore, could it be prosecuted in detail and with quantitative exactness, might be expected to throw some light upon the complex problem of protein constitution.

Unfortunately, in the medley of substances presented by a tryptic digest few amino-acids can be estimated with anything like precision. There are, indeed, but three—tyrosine, tryptophan and glutamic acid—of which it has been possible hitherto to follow the appearance by quantitative methods. Brown and Millar [1906] and, later, Plimmer and Eaves [1913], found that tyrosine was completely liberated in from 1 to 6 hours of tryptic digestion. In the experiments of Abderhalden and Reinbold [1905] and Abderhalden and Voegtlin [1907], its appearance was less rapid, but within two to three days equally complete. The behaviour of tryptophan would seem to be similar; for Ragins [1928] employing a colorimetric method of estimation, found that of the total amount potentially present from one-half to three-fourths was set free within 1 hour and the remainder within 1 to 5 days. On the other hand Fürth and Lieben [1920], who used a different colorimetric method, came to

[1] The experimental data of this paper are taken from a thesis presented by James A. Dauphinee in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Toronto.

[2] Throughout this paper we use the term "trypsin" in its original sense as a collective designation for the protein- and peptide-splitting enzymes of the activated pancreatic secretion. These, it has within recent years been shown [Waldschmidt-Leitz and Harteneck, 1925, 1, 2; Waldschmidt-Leitz, Schäffner and Grassmann, 1926; Waldschmidt-Leitz, Balls and Waldschmidt-Graser, 1929; Waldschmidt-Leitz and Purr, 1929], include no less than four distinct and highly specific hydrolysing agents—a proteinase, two polypeptidases and a dipeptidase. The crude preparations which we (and others before us) have employed may have contained any or all of these enzymes. In the effects which we are about to describe the proteinase probably played the predominant part; for it alone is capable of acting upon native proteins. How far its action may have been modified or supplemented by accompanying peptidases must be left for the present uncertain.

the conclusion that tryptophan is liberated very slowly and incompletely. Glutamic acid has been shown to behave quite differently [Abderhalden and Reinbold, 1905; Abderhalden and Voegtlin, 1907]; its liberation is a very gradual process, and the maximum yield, which may not be attained for 2 to 3 weeks, falls decidedly short of complete hydrolysis.

With respect to the remaining amino-acids little more is known than that some (like proline and phenylalanine) are not released at all by trypsin acting alone [Fischer and Abderhalden, 1903; Abderhalden and Reinbold, 1905], and that the behaviour of others (including alanine, valine, leucine, aspartic acid and the hexone bases) resembles more or less closely that of glutamic acid [Abderhalden, 1922, p. 464].

The work now to be reported was planned in the hope of adding arginine to the small list of amino-acids, concerning the liberation of which by trypsin we have precise quantitative information. That free arginine does actually appear in tryptic digests was shown by Kutscher [1898, 1899] and by Kossel and Mathews [1898]. Its presence has been noted also among the products arising from the proteolytic action of certain enzymes of the spleen [Leathes, 1902; Cathcart, 1905]. On the other hand there are on record a number of observations (to be cited later) which suggest that part of the arginine of proteins resists indefinitely the action of trypsin. Quantitative data with respect to either aspect of the case are entirely lacking; and, although it has been generally thought that arginine is liberated rather slowly, there is no experimental basis for this belief. Our own experiments indicate that as a matter of fact the liberation of arginine, though never complete, may sometimes be just as rapid as that of tryptophan or tyrosine.

Methods.

(*a*) *General.*

The general plan of our experiments was to bring an approximately 10 % solution of protein to a p_H of 8·2 (or thereabout) and a temperature of 37°, and to treat it with enough of a commercial trypsin preparation to give a concentration of 0·4 %. The mixture (protected by toluene against bacterial contamination) was allowed to digest at 37°, while at stated intervals samples were removed and boiled. On each sample we then determined total nitrogen (by Kjeldahl), free amino-nitrogen (by the micro-method of Van Slyke), ammonia (by treatment with potassium carbonate and aeration) and arginine (by the method discussed below). The total arginine content of each protein studied was determined on a separate sample hydrolysed by boiling for 4–24 hours with 20 % hydrochloric acid [Hunter and Dauphinee, 1930]. In some cases we determined also the free amino-nitrogen of both the original and the completely hydrolysed protein; in others (as will be indicated in the tables) we accepted for these the values given in the literature.

Certain variations of detail in treatment and in sampling, necessitated by

the different behaviour of the individual proteins, will be mentioned later at appropriate points.

(*b*) *Determination of arginine.*

In determining the free arginine of each sample of digest we employed the method of Jansen [1917, 1, 2], in which the arginine is hydrolysed by the enzyme arginase and the resulting urea is determined by the use of urease. Elsewhere [Hunter and Dauphinee, 1930], we have demonstrated the accuracy of this procedure and have described in detail its application to the determination of arginine in completely hydrolysed proteins. In that application it was found convenient to use, as the source of arginase, a crude glycerol extract of liver clarified by a brief period of heating at 60°. As such an extract is itself capable of liberating arginine from partly digested proteins, it was deemed necessary in the present investigation so to treat it that, retaining as much as possible of its power to decompose arginine, it should lose all or most of its proteolytic activity. A sufficiently satisfactory method of reaching this result was found in the use of aluminium hydroxide.

The aluminium hydroxide was prepared according to the directions of Willstätter and Kraut [1923], and was used only after it had been converted by long standing into the highly active C_γ modification [Willstätter, Kraut and Erbacher, 1925]. Preliminary experiments showed that this adsorbent, applied at p_H 5·0, would remove from our liver extracts practically all the protease, but comparatively little of the arginase. The latter, however, is subject at the p_H specified to a rather rapid auto-destruction. The following, therefore, was the method finally adopted to obtain active arginase preparations suitable for our purpose[1]. 30 cc. of an ice-cold crude extract of liver, prepared in the manner described by Hunter and Dauphinee [1930, pp. 632–3], are treated with enough *N* HCl (usually about 0·9 cc.) to bring the p_H to approximately 5·0. 15 cc. of an 11·5 % suspension of alumina C_γ are added, and the mixture is quickly centrifuged. The supernatant liquid is treated with another 15 cc. of the alumina suspension, and centrifuged again. The second supernatant liquid is then adjusted to neutrality by addition of the required amount of *N* NaOH. To minimise the destruction of arginase all these operations are carried out as rapidly, and as nearly at the freezing point, as possible. Much time may be saved by determining beforehand the exact quantities of *N* HCl and *N* NaOH that will produce the p_H changes desired. The procedure described should yield about 42 cc. of a product containing from 30 to 40 units[2] of arginase per cc.

In applying the purified arginase to the determination of arginase in tryptic digests we followed a procedure the same in essence as that described

[1] That aluminium hydroxide may be applied to the preparation of protease-free arginase has been shown already by Edlbacher and Simons [1927]; but, although their results are not inconsistent with ours, their method of utilising them was quite different.

[2] The unit here meant is that defined and used by Hunter and Dauphinee [1930, p. 630].

by Hunter and Dauphinee [1930, pp. 649–653] as suitable for the direct determination of the substance in total hydrolysates without previous removal of either humin or ammonia. The samples to be analysed, measuring either 5 or 10 cc., were treated with a drop of phenolphthalein indicator and enough *N* NaOH to give a permanent light pink colour. To each were then added at least 50 units of arginase (usually 2 cc. of alumina-treated extract). The further addition of a buffer was not found necessary. The enzyme was allowed to act for 10 to 12 hours at 37°[1], and the urea formed was then determined by the urease method of Van Slyke and Cullen. Each determination was performed in duplicate, and was accompanied by the appropriate blank controls. For details concerning these reference may be made to the earlier paper just cited.

(*c*) *Trypsin controls.*

The "trypsin" added to each solution of protein undergoes itself a fairly extensive digestion, with liberation of arginine, so that control experiments are always necessary. In these controls 2 g. of each trypsin preparation used, together with enough Na_2CO_3 to give p_H 8·2, were dissolved in 500 cc. of water at 37°. The total nitrogen of the solution was determined on a 15 cc. sample. Samples of 50 cc. were withdrawn at convenient intervals, heated to boiling, and cooled. Arginine (and ammonia) were then determined on 15 cc. portions, amino-nitrogen on 2 cc. portions, of each. The results were calculated to 100 cc. of the original solution.

Materials.

The proteins or protein derivatives included in the investigation were gelatin, caseinogen, edestin, egg-albumin, fibrin and Witte-peptone. For the first of these we used the Bacto-gelatin of the Digestive Ferments Company. The egg-albumin and fibrin bore the label of Merck. The caseinogen was prepared from a commercial product (already fairly pure) by dissolving in dilute NaOH, precipitating with acetic acid, repeating the process, and drying the final product with alcohol and ether. The edestin was extracted from defatted hempseed with warm 5 % sodium chloride, and precipitated by pouring the extract into 10 volumes of water; the product was redissolved and reprecipitated thrice, washed free from salt, and dried with alcohol and ether.

For the digestion of these proteins we used two different highly active brands of commercial "trypsin." In the description of the experiments these will be distinguished as trypsin-D and trypsin-P.

[1] The conditions adopted would have ensured the quantitative decomposition of several times as much arginine as any of our samples could have contained.

Experiments and results.

(*a*) *Experiments with caseinogen.*

Exp. 1. Ten successive 5 g. portions of caseinogen were ground in a mortar, each with 50 cc. of 0·06*N* NaOH. The solutions were combined, and the mixture was treated with so much 5*N* NaOH that it gave a just perceptible pink colour with phenolphthalein (p_H 8·2). 500 cc. of the clear solution of caseinogen thus obtained were placed in a flask, brought in a water thermostat to a temperature of 37°, and at a given instant treated with 2 g. of trypsin-P. The trypsin was quickly dissolved by shaking, 10 cc. of toluene were added, and the flask was returned to the thermostat. At intervals of 1, 3, 9 and 24 hours, and of 3, 7 and 14 days, a 50 cc. portion of the well shaken digest was pipetted into a 100 cc. volumetric flask containing 8 drops of 20 % HCl, 30 cc. of water were added, and the mixture was heated until the separated tyrosine had dissolved and the enzyme had been destroyed. After cooling to room temperature the solution was neutralised and made up to 100 cc.

For analysis 3 cc. were taken for the determination of total nitrogen, 10 cc. for ammonia and 10 cc. for arginine. For the free amino-nitrogen 4 cc. of the first three samples, or 2 cc. of the later ones, were further diluted to 10 cc., and the determination was carried out on duplicate or triplicate portions of 1 cc.

The results are shown in Tables I and II. The figures in the last 2 columns of Table II—recorded as "percentage hydrolysis of peptide" or "arginide links"[1]—are calculated according to the formula $\frac{100\,(A - A_0)}{A_1 - A_0}$, in which A signifies the observed value at any given time, A_0 the value for the intact protein before hydrolysis, and A_1 the value after complete hydrolysis[2].

Table I. *Tryptic digestion of caseinogen. Exp. I.*

(Original data for 10 cc. portions of the diluted samples, each portion containing 68·03 mg. N, and corresponding to 5 cc. of the original digest.)

		Mg. ammonia-N				
Time of digestion	Mg. free amino-N	(*a*) in untreated sample (amide blank)	(*b*) in sample treated with arginase and urease	(*c*) yielded by blank controls*	(*d*) derived from liberated arginine (*b*)–(*c*)	Mg. arginine-N liberated†
1 hr.	13·37	0·28	1·93	0·86	1·07	2·17
3 hrs.	17·67	0·35	2·44	1·04	1·40	2·84
9 ,,	22·67	0·59	3·07	1·31	1·76	3·57
24 ,,	25·67	0·73	3·36	1·50	1·86	3·78
3 days	28·70	1·27	4·28	2·26	2·02	4·10
7 ,,	31·20	1·85	4·76	2·68	2·08	4·22
14 ,,	32·00	2·52	5·24	3·06	2·18	4·43

* This includes the "amide blank" of column (*a*). See Hunter and Dauphinee [1930].

† Calculated by doubling the quantity in column (*d*) and adding the empirical correction of 1·5 % [Hunter and Dauphinee, 1930].

[1] Here, and elsewhere throughout this paper, these expressions are used solely as a descriptive convenience, and without prejudice to the question whether other than peptide links play any part in the structure of the protein molecule.

[2] In the case of arginine A_0 of course is always zero.

Table II. *Tryptic digestion of caseinogen.*

Exp. 1. Trypsin-P 0·4 %. Total N in 100 cc. digest = 1·361 g.; of which substrate-N = 1·314 g.
Exp. 2. Trypsin-D 0·4 %. Total N in 100 cc. digest = 1·576 g.; of which substrate-N = 1·529 g.
Exp. 3. Trypsin-D 0·8 %. Total N in 100 cc. digest = 1·296 g.; of which substrate-N = 1·202 g.

(All results are calculated to 100 cc. digest.)

Time of digestion	Mg. free amino-N: in whole digest	Mg. free amino-N: in trypsin control	Mg. free amino-N: derived from substrate	Mg. arginine-N liberated: in whole digest	Mg. arginine-N liberated: in trypsin control	Mg. arginine-N liberated: derived from substrate	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
					Exp. 1.					
0 hrs.	—	—	—	—	—	—	5·73*	0·00	0·0	0·0
1 ,,	267·4	18·0	249·4	43·4	1·1	42·3	19·0	3·22	19·9	40·4
3 ,,	353·4	19·5	333·9	56·8	3·5	53·3	25·5	4·06	29·6	50·9
9 ,,	453·4	22·0	431·4	71·4	4·5	66·9	32·8	5·09	40·6	63·8
24 ,,	513·4	24·5	488·9	75·6	4·7	70·9	37·2	5·40	47·2	67·7
3 days	574·0	25·6	548·4	82·0	5·0	77·0	41·7	5·86	54·0	73·4
7 ,,	624·0	28·1	595·9	84·4	5·0	79·4	45·4	6·04	59·4	75·7
14 ,,	640·0	28·2	611·8	88·5	5·7	82·8	46·6	6·30	61·3	79·0
Complete hydrolysis	—	—	—	—	—	—	72·4*	7·98	100·0	100·0
					Exp. 2.					
14 days	760·7	31·3	729·4	105·6	6·9	98·7	47·7	6·45	63·0	80·9
21 ,,	—	—	—	109·6	6·9	102·7	—	6·72	—	84·2
35 ,,	—	—	—	110·9	6·8	104·1	—	6·81	—	85·3
					Exp. 3.					
3 hrs.	425·0	43·6	381·4	71·4	11·6	59·8	31·7	4·97	39·0	62·3
24 ,,	560·0	50·0	510·0	86·0	13·2	72·8	42·4	6·06	54·9	76·0
7 days	645·0	58·6	586·4	91·0	13·8	77·2	48·8	6·42	64·6	80·4
14 ,,	694·0	62·6	631·4	93·0	13·8	79·2	52·5	6·59	70·2	82·6

* Hunter and Smith [1925].

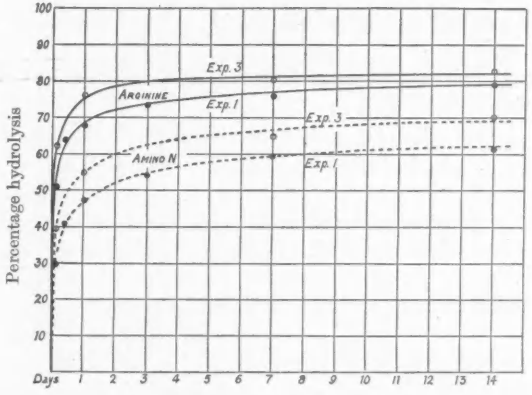

Fig. 1. Tryptic digestion of caseinogen. Liberation of arginine and amino-nitrogen under different concentrations of enzyme.

A graphical representation of the results of Exp. 1 will be found in Fig. 1, in which percentage hydrolysis is plotted against time. From this curve, as from the data of the tables, it appears that the initial velocity of arginine liberation in the case of caseinogen is very great. In a relative sense it surpasses even the general rate of polypeptide cleavage. Thus in the first hour,

during which only 20 % of the total peptide linkages were split, as much as 40 % of the total available arginine was set free. In 3 hours the proportion freed had risen to a half, in 24 hours to two-thirds. From this point the rate of liberation rapidly fell off, until during the second week hydrolysis seemed to have all but come to a standstill. On the 14th day, when the experiment was terminated, the proportion of originally bound arginine which had been freed was almost 80 %. The free amino-nitrogen in the meantime had risen to only 61 % of the possible maximum.

The question at once presented itself, whether with the liberation of just four-fifths of the total arginine the reaction had reached a definite and reproducible end-point. In order to test this Exps. 2 and 3 were carried out.

Exp. 2. This was a repetition of Exp. 1 with the following differences. (1) A new preparation of caseinogen was employed. (2) Trypsin-D was substituted for trypsin-P. (3) Arginine was determined only at the end of 2, 3 and 5 weeks of digestion. (4) Amino-nitrogen was determined only at the end of the 2nd week.

The results are shown beside those of Exp. 1 in Table II. It will be noted that at the 14th day both amino-N and arginine had reached practically the same values as in Exp. 1, so that the two trypsins used were of almost identical potency. In the 3 weeks following the free arginine did definitely increase, but the rate of increase, especially in the last 14 days, was exceedingly slow. The proportion of arginine ultimately liberated was 85 %.

Exp. 3. In this experiment we followed again the same general procedure as before, but used enough trypsin-D to give a concentration of 0·8 instead of 0·4 %. Samples were removed at 3 and 24 hours, and at 7 and 14 days. The results are again incorporated in Table II, and in Fig. 1 are compared graphically with those of Exp. 1.

As was to be expected, the higher concentration of enzyme led to a more rapid proteolysis. More than 60 % of the arginine was free at the end of 3 hours, and as much as 75 % at the end of 24. Later the arginine curves of the two experiments converge, and at the end of 2 weeks the higher enzyme concentration has raised the total amount of arginine liberated at the most by some 4 %.

Exps. 2 and 3 do not provide an altogether unambiguous answer to the question which they were designed to settle. In each the percentage of arginine liberated did reach an appreciably higher level than in Exp. 1. But in each case the effect was small, and its significance is doubtful. The enzyme preparations used contained almost certainly (see p. 1128, footnote), along with their trypsin, a certain proportion of ereptic enzymes. For the main effect, culminating apparently in the second week of digestion, the former, as the predominant factor, may be presumed to be responsible; the succeeding much more gradual increase of free arginine reveals perhaps the influence, at what was doubtless a much lower concentration, of the latter. Possibly, also, the slight alkalinity of the digests was in itself sufficient to bring about, at 37°,

a slow continuous hydrolysis. Such considerations, hypothetical as they are, lead us to question the importance of fractional increases taking place only upon prolonged incubation. On the whole, therefore, we are inclined to interpret our results as indicating that the activated protease of the pancreas sets free just 80 % of the total arginine of caseinogen[1]. We admit the somewhat arbitrary nature of this conclusion, and the desirability of testing it by further experiments with a purified enzyme.

(*b*) *Experiments with gelatin.*

Exp. 4. 50 g. of gelatin were dissolved by warming in 500 cc. of water, and the reaction of the solution was adjusted with NaOH to p_H 8·2. Of this solution 500 cc. were brought to 37°, and treated with 2·0 g. of trypsin-D. From this point the procedure followed was the same as in Exp. 1 with caseinogen. The only difference of detail was that in the present case there took place, of course, no separation of tyrosine.

Exp. 5. This was a repetition of Exp. 4 with the difference that only arginine determinations were made, and these only after 2, 3 and 5 weeks of digestion. The object was simply to check the result of the first experiment with respect to the maximum amount of arginine liberated.

In reporting the results of these, as of all subsequent experiments, we omit, for the sake of brevity, the original analytical data (such as were shown for caseinogen in Table I), as well as the corrections (exemplified in Table II)

Table III. *Tryptic digestion of gelatin.*

(Corrected values for 100 cc. of digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
		Exp. 4. Total substrate-N = 1·363 g.				
0 hrs.	43·1	—	3·16	0·00	00·0	00·0
1 ,,	170·8	97·6	12·53	7·16	12·8	45·2
3 ,,	222·9	113·6	16·35	8·34	19·5	52·6
9 ,,	262·4	129·4	19·25	9·49	23·8	59·9
24 ,,	336·5	142·8	24·69	10·48	31·8	66·1
3 days	391·4	153·3	28·71	11·25	37·7	71·0
7 ,,	416·9	165·7	30·58	12·16	40·5	76·7
15 ,,	451·8	170·9	33·15	12·54	44·3	79·1
21 ,,	466·8	175·8	34·25	12·90	46·0	81·4
63 ,,	—	180·5	—	13·24	—	83·5
Complete hydrolysis	—	—	70·8	15·85	100·0	100·0
		Exp. 5. Total substrate-N = 1·491 g.				
14 days	—	189·3	—	12·70	—	80·1
21 ,,	—	196·4	—	13·17	—	83·1
35 ,,	—	202·6	—	13·59	—	85·7

[1] Some years ago we reported briefly preliminary experiments not only with caseinogen but also with gelatin [Hunter and Dauphinee, 1925; Hunter, 1925]. In both cases we obtained a maximum arginine yield of only two-thirds instead of, as now, four-fifths. The records of these early experiments were unfortunately lost in a laboratory fire, and we are unable at present to offer an explanation of the discrepancy.

which have to be made for the self-digestion of the trypsin. Table III contains therefore the results of Exps. 4 and 5 as calculated for 100 cc. of digest and already corrected by the corresponding trypsin controls.

A comparison of Tables II and III shows that, if the increase of free amino-nitrogen be taken as the measure of proteolysis, gelatin will appear to be digested more slowly and less completely than caseinogen. With respect, on the other hand, to the rate and the extent of arginine liberation the behaviours of these two proteins are practically identical, so that the curve of arginide hydrolysis given for caseinogen in Fig. 1 would serve almost equally well for gelatin. The very slightly different curve actually obtained for the latter appears with others in Fig. 2. Its initial part is produced again, on a different time scale and for a special purpose, in Fig. 3. We have thought it unnecessary to complicate Fig. 2 by introducing into it curves of peptide hydrolysis; but for the case of gelatin such a curve accompanies the curtailed arginine curve of Fig. 3.

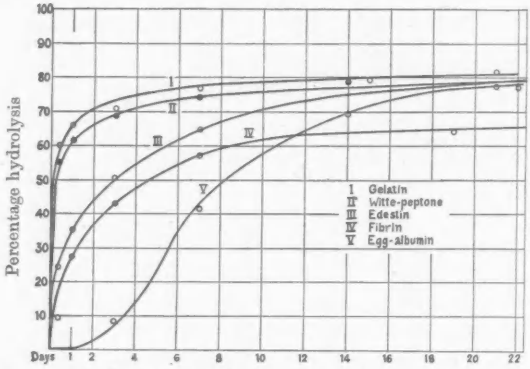

Fig. 2. Liberation of arginine in tryptic digestion of various proteins.

Exps. 4 and 5 are in accord in suggesting that the end-result of the action of trypsin on gelatin is the liberation of four-fifths of its total content of arginine.

(*c*) *Experiments with edestin.*

Exp. 6. 25 g. of edestin were dissolved by grinding in a mortar with 250 cc. of 0·25 % sodium carbonate, and by the cautious addition of HCl the reaction of the solution was so adjusted that it gave a just perceptible pink colour with phenolphthalein. The solution having then been brought to 37° was at a given instant treated with 1 g. of trypsin-P and 5 cc. of toluene. Since the earlier samples of this digest coagulated upon heating, the procedure of sampling and analysis hitherto employed was modified as follows. At selected intervals portions of 25 (or for those taken at the 29th and 35th days, 10) cc. were removed, and treated, for the immediate inhibition of tryptic action, with 4 (or 2) drops of 20 % HCl. The samples taken at the 1st and 3rd hours

were left undiluted, the others were made up with water to a volume of 50 cc. Aliquots of 5 cc. each were then measured into urea tubes, heated in boiling water for the final destruction of the enzyme, and used, without separation of any resulting coagulum, for the determination of ammonia and of arginine. Free amino- and total nitrogen were determined on suitable unheated aliquots.

The results were calculated to 100 cc. of digest, and in that form, after suitable correction for the added trypsin, are exhibited in Table IV. The rate of liberation of arginine is shown graphically in Fig. 2.

Table IV. *Tryptic digestion of edestin.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N		Percentage hydrolysis	
			as free amino-N	as arginine-N	of peptide links	of arginide links
		Exp. 6. Total substrate-N = 1·626 g.				
0 hrs.	—	—	1·8*	0·00	0·0	00·0
1 „	111·2	58·2	6·84	3·58	8·3	13·4
3 „	175·5	80·1	10·8	4·93	14·8	18·5
8 „	239·2	105·9	14·7	6·51	21·1	24·4
24 „	283·5	153·6	23·6	9·41	35·7	35·3
3 days	584·4	219·1	—	13·47	—	50·5
7 „	656·9	281·6	40·4	17·32	63·3	64·9
22 „	801·8	334·5	49·3	20·57	77·9	77·0
29 „	—	357·7	—	22·00	—	82·4
35 „	863·5	360·5	53·1	22·17	84·1	83·0
Complete hydrolysis	—	—	62·8†	26·7	100·0	100·0

* Van Slyke and Birchard [1914]. † Van Slyke [1912].

If the data of Table IV be compared with those of Tables II and III it will be evident that the behaviour of edestin under the action of trypsin differs considerably from that of caseinogen or gelatin. From both of the latter the amino-groups in general and the arginine in particular are liberated with a speed which may almost be described as explosive. Under identical conditions the hydrolysis of edestin is in each respect much more gradual. The contrast between edestin and gelatin is made very evident in the curves of Fig. 2. It was equally conspicuous in certain earlier experiments, of shorter duration than the present, which have been briefly reported elsewhere [Hunter, 1925; Hunter and Dauphinee, 1925]. The difference is therefore not accidental, but truly characteristic.

It was 3 or 4 weeks before the liberation of arginine in the present experiment came to an apparent standstill. The proportion then free was approximately 80 %. In this respect there would appear to be little, if any, difference between edestin and the two proteins previously discussed[1].

[1] It ought, however, to be mentioned that in another experiment, conducted upon a rather small scale and with a specimen of unknown origin, edestin appeared to yield a distinctly higher proportion of free arginine.

(*d*) *Experiments with egg-albumin.*

Exp. 7. 500 cc. of an approximately 10 % filtered solution of egg-albumin were digested at 37° and p_H 8·0 with 2 g. of trypsin-P. The sampling and the analyses were carried out in the same way as with edestin, except that none of the samples was diluted (Table V and Fig. 2).

Table V. *Tryptic digestion of egg-albumin.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
Exp. 7. Total substrate-N = 1·113 g.						
0 hrs.	54·8	—	4·92	0·00	0·00	0·00
3 „	56·1	1·76	5·04	0·16	0·16	1·54
9 „	66·0	—	5·93	—	1·35	—
24 „	89·9	—	8·08	—	4·24	—
3 days	168·6	9·61	15·2	0·86	13·8	8·32
7 „	432·0	47·8	38·8	4·29	45·5	41·4
14 „	641·8	79·6	57·7	7·15	70·8	69·2
21 „	685·8	89·3	61·6	8·02	76·0	77·4
37 „	701·8	97·0	63·1	8·72	78·0	84·1
Complete hydrolysis	—	—	79·5*	10·36	100·0	100·0

* Van Slyke [1912].

The peculiar course of the digestion in this experiment exemplifies the well-known resistance of uncoagulated egg-albumin to the initial attack of trypsin [Vernon, 1904; Bayliss, 1908, p. 67][1]. This resistance has usually been ascribed to the influence of an antitrypsin, and it has been supposed that only as the antitrypsin disappears does the trypsin itself gradually become effective. The curve of proteolysis is in this way delayed and distorted. Under the existing conditions the rate of liberation of arginine, from the 4th day onward, is represented by a curve which shows a general resemblance to that obtained with edestin. The final result is the liberation, once again, of approximately four-fifths of the total arginine. To this figure it would be unwise in the present instance to attach too much significance; for the albumin used was probably far from pure.

(*e*) *Experiments with fibrin.*

Exp. 8. 50 g. of dried fibrin were reduced to powder, suspended in 500 cc. of water, brought to a temperature of 37°, and treated with 1 g. of sodium carbonate, 2 g. of trypsin-P and 10 cc. of toluene. At the end of the 1st hour of digestion much of the fibrin was still in suspension. The first sample analysed was taken at the 3rd hour, when practically all of the fibrin had gone into solution. The early samples coagulated on heating, and were treated therefore in the same way as those of edestin and egg-albumin.

[1] For further references to the literature of this phenomenon see Bateman [1916].

Exp. 9. This was a repetition of Exp. 8 with these differences—a new lot of fibrin was taken, trypsin-D replaced trypsin-P, and samples were taken only after 2, 3 and 5 weeks (Table VI and Fig. 2).

Table VI. *Tryptic digestion of fibrin.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N: as free amino-N	Percentage of total substrate-N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
Exp. 8. Total substrate-N = 1·215 g.						
0 hrs.	—	—	1·6*	0·00	0·0	0·00
3 „	142·9	11·9	11·8	0·98	13·0	6·85
9 „	240·4	16·2	19·8	1·33	23·2	9·29
24 „	349·5	47·7	28·8	3·93	34·7	27·5
3 days	484·4	74·6	39·9	6·14	48·8	42·9
7 „	631·9	99·3	51·9	8·17	64·1	57·1
19 „	691·8	111·2	56·9	9·15	70·5	63·9
29 „	—	114·5	—	9·42	—	65·8
Complete hydrolysis	—	—	80·0*	14·31	—	—
Exp. 9. Total substrate-N = 1·231 g.						
14 days	—	114·5	—	9·30	—	65·0
21 „	—	111·9	—	9·09	—	63·5
35 „	—	109·3	—	8·88	—	62·1

* Levene and Bass [1929].

Whether one judges the rate of proteolysis by the increase of free amino-nitrogen or by the liberation of arginine, it will appear from Exp. 8 that fibrin is digested much less rapidly than gelatin or caseinogen. In the gradual progress of the reaction it rather resembles edestin. Nevertheless the proportion of peptide groups ultimately hydrolysed is very high. On this criterion fibrin would in fact seem to be quite as susceptible to the hydrolytic action of trypsin as any of the proteins already examined. It is otherwise with respect to the liberation of arginine; with fibrin the fraction liberated is decidedly smaller. Both experiments are in agreement in setting it at about two-thirds. (The apparent diminution of free arginine after the second week in Exp. 9 is probably accidental.)

(*f*) *Experiments with Witte-peptone.*

The experiments with fibrin having shown that a large fraction of its arginine resisted liberation by trypsin, it seemed of interest to ascertain whether the proteoses and peptones derivable from it would exhibit in this respect any greater susceptibility. As a preliminary test of this possibility we carried out, therefore, the following experiments with Witte-peptone (Table VII and Fig. 2).

Exp. 10. An approximately 10 % filtered solution of Witte-peptone was digested at 37° and p_H 8·2, with 0·4 % of trypsin-P. In sampling this digest and in analysing the samples we followed exactly the same procedure as in Exp. 1 with caseinogen.

Exp. 11. A different lot of Witte-peptone was digested under the same conditions with trypsin-D; but the samples were taken only at the end of 2, 3 and 5 weeks, and were analysed for arginine alone.

Table VII. *Tryptic digestion of Witte-peptone.*

(Corrected values for 100 cc. digest.)

Time of digestion	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total substrate-N		Percentage hydrolysis	
			as free amino-N	as arginine-N	of peptide links	of arginide links
		Exp. 10. Total substrate-N = 1·325 g.				
0 hrs.	184·0	0·0	13·9	0·00	0·0	0·0
1 „	338·0	51·2	25·6	3·86	18·9	27·9
3 „	430·5	79·7	32·6	6·01	30·0	43·4
9 „	468·0	101·1	35·5	7·62	34·7	55·0
24 „	555·5	111·0	42·0	8·52	45·3	61·5
3 days	604·4	126·5	45·8	9·53	51·3	68·8
7 „	641·9	136·7	48·6	10·30	55·8	74·4
14 „	671·7	144·9	50·8	10·92	59·3	78·8
28 „	671·7	146·6	50·8	11·05	59·3	79·8
Complete hydrolysis	—	—	76·1	13·85	100·0	100·0
		Exp. 11. Total substrate-N = 1·465 g.				
14 days	—	161·2	—	11·01	—	79·5
21 „	—	167·7	—	11·45	—	82·7
35 „	—	170·5	—	11·64	—	84·1

(*g*) *The liberation of arginine in acid hydrolysis.*

The hydrolysis of proteins by acids is known to follow in several respects a different course from that of tryptic digestion. It seemed therefore worth while to compare, in at least one instance, the curves of arginine liberation in these two processes. With this object we carried out the following experiment, in which gelatin was subjected to the action of *N* HCl at 100°. The conditions were so chosen that the proportion of arginine liberated within 24 hours would not be greatly different from that liberated within the same period by 0·4 % of trypsin at 37°.

Exp. 12. A flask containing 300 cc. of 2*N* HCl was immersed in a boiling water-bath, and allowed to come to temperature equilibrium. There were then added to it 300 cc. of a 10 % solution of gelatin already raised to the boiling point. The flask was fitted with a reflux condenser, and hydrolysis was allowed to proceed, at a temperature of approximately 100°, for a period of 80 hours. At given intervals samples were removed from the flask, added to a quantity of 5*N* NaOH calculated to neutralise the acid, cooled, and made up to a convenient volume. Suitable aliquots were then used for the determination of total nitrogen, free amino-nitrogen, and arginine[1]. The results as calculated to 100 cc. of hydrolysate are shown in Table VIII. In Fig. 3 they are

[1] The determination of arginine involved of course a determination of ammonia. But as the ammonia results merely confirmed those of Pittom [1914] and Vickery [1922] in showing that the amide-N of proteins is completely liberated at a very early stage of acid hydrolysis, we have not recorded these separately.

exhibited graphically side by side with the corresponding parts of the curves for the tryptic digestion of gelatin (Exp. 4).

Table VIII. *Acid hydrolysis of gelatin. Exp. 12.*

(The data are for 100 cc. hydrolysate, containing 693·8 mg. total nitrogen.)

Time of hydrolysis	Mg. free amino-N	Mg. arginine-N liberated	Percentage of total N: as free amino-N	Percentage of total N: as arginine-N	Percentage hydrolysis: of peptide links	Percentage hydrolysis: of arginide links
0 hrs.	—	—	3·16	0·00	0·0	0·0
1 ,,	154·9	21·1	22·3	3·04	28·3	19·2
2 ,,	217·2	25·0	31·3	3·60	41·6	22·7
6 ,,	304·3	38·6	43·8	5·56	60·1	35·1
18 ,,	373·3	65·8	53·8	9·48	74·9	59·8
32 ,,	400·3	75·1	57·7	10·82	80·6	68·3
80 ,,	440·8	92·1	63·5	13·27	89·2	83·7
Total hydrolysis	—	—	70·8	15·85	100·0	100·0

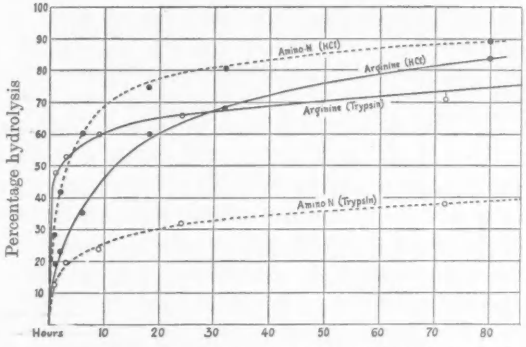

Fig. 3. Comparison of tryptic digestion with acid hydrolysis of gelatin.

The curves reveal at once decided differences between the two types of hydrolysis. As has been noted already, the effect of trypsin upon gelatin is to liberate arginine at a speed greatly in excess of the general rate of hydrolysis as measured by the increase of free amino-groups. Under the action of hydrochloric acid this relation is reversed. Another difference is that the initial rate of arginine liberation is much lower with the acid than with the enzyme. On the other hand the action of the enzyme slackens almost as suddenly as it starts, and is limited (as has been shown) to a partial hydrolysis; whereas that of the acid falls off much less abruptly, and continues until the liberation of arginine and other amino-acids is complete. The consequence is that after a time (in the present experiment about 30 hours) the two arginine curves cross one another, and the acid hydrolysate comes to contain the greater proportion of free arginine.

A full interpretation of these differences is for the present impossible; but their existence emphasises the selective character of enzymic, as contrasted with acid, hydrolysis.

Discussion.

The experiments which have been described demonstrate the liberation during tryptic digestion of a substance (or substances) susceptible to the action of arginase.

Up to this point it has been tacitly assumed that this substance is simple arginine. The correctness of this assumption, it must now be stated, is by no means certain. Although arginase is without action upon α-methylarginine, δ-methylarginine, dibenzoylarginine or arginine methyl ester, it liberates urea from α-monobenzoylarginine. Felix, Müller and Dirr [1928] conclude accordingly that any change in the guanidine or the carboxyl group of arginine, or the introduction into its amino-group of an alkyl radical, protects it from the action of arginase; but that the enzyme can still attack those derivatives in which the only substitution is that of one hydrogen of the amino-group by an acyl radical. If this generalisation is correct, arginase might be expected to act upon certain peptides of arginine—upon all those, namely, in which the arginine is bound only through its amino-group. Peptides of this type might conceivably enough be liberated during the course of tryptic digestion. It is therefore not impossible that the "arginine" determined in our experiments was still, in whole or in part, in a state of combination.

This argument rests of course upon a hypothesis, and there exists at present no direct evidence in support of the proposition to which it leads. The idea that peptide-bound arginine may in certain cases be susceptible to the action of arginase seemed indeed to be confirmed by the observation of Edlbacher and Bonem [1925] that Fischer's "arginylarginine" yields to the enzyme, as the theory would predict, one half of its total guanidine-nitrogen as urea. Unfortunately, as Zervas and Bergmann [1928] have shown, the supposed dipeptide of arginine is really an anhydride, and its apparent susceptibility to arginase was due, in all probability, to an admixture of free arginine. We possess as yet, therefore, no actual proof that any arginine peptide whatsoever is attacked by arginase.

Still less have we any proof that susceptible arginine peptides do actually appear under the action of trypsin. We have ourselves been unable, in spite of many attempts, to separate from tryptic digests any product, other than arginine itself, which would yield urea when treated with arginase. The only evidence which we have secured for the presence of such products is contained in the observation that flavianic acid, which forms a nearly insoluble compound with arginine, does not precipitate from a tryptic digest all of the material which is capable of reacting with arginase. Thus, in a particular 2-hour digest of gelatin the arginine determined by arginase amounted to 9·05 % of the total nitrogen, while the nitrogen precipitated by flavianic acid was only 7·24 %. In another digest, 18 days old, arginase indicated 12·25 % of arginine-nitrogen, flavianic acid only 11·32 %. In the first case therefore 19·8 %, in the second 7·6 %, of the arginine determined by arginase failed

to be precipitated by flavianic acid. This reagent was found to give in each case complete recovery of added arginine. The results might therefore be interpreted as indicating that the material susceptible to arginase, especially perhaps that of the earlier stages of digestion, included something other than free arginine. Unfortunately we cannot at present exclude the possibility that they depend simply upon an enhancement of the solubility of arginine flavianate by the more complex products of digestion.

A complete answer to the question at issue must be left to future work. In the meantime we must be content to recognise that what we have determined in our experiments may be either simple arginine, or some particular type of arginine-complex, or a mixture of both. In any case it is arginine which has been liberated from those connections which, in the intact protein, protect it from the action of arginase.

The rapidity with which this liberation takes place varies, as our experiments have shown, with the nature of the substrate. It is greatest in the cases of caseinogen and the two protein derivatives, gelatin and Witte-peptone. In all of these a few hours of digestion suffices to detach half at least of the total arginine from the parent molecule. The arginine of edestin, fibrin and egg-albumin is set free much more slowly, the liberation of 50 % requiring 3, 4½ and 8 days respectively. These differences are characteristic, but there is nothing about them that is specific for arginine. Very similar differences were found by Ragins [1928] in the rate of liberation of tryptophan from different proteins. Moreover, with each of the proteins studied in the present paper the curve of arginide hydrolysis did but parallel that of general peptide hydrolysis. For the cases of caseinogen and gelatin this is made evident in Figs. 1 and 3 respectively; and if the data for free amino-N had been plotted in Fig. 2, it would have been seen to be equally true for the others. The hydrolysis of the arginide links proceeds, therefore, simply *pari passu* with that of all the other peptide bonds actually susceptible, in any given instance, to the action of trypsin. This would indicate that differences in the rate of liberation of arginine are determined by differences (perhaps physical rather than chemical) in the general character of the molecule—not, as might otherwise have been supposed, by specific differences in the mode of union of arginine itself.

But, although the arginine curve and that for free amino-nitrogen run always parallel, it is only in the cases of edestin and egg-albumin that they show an approach to exact coincidence. With caseinogen (Fig. 1), with Witte-peptone and, still more conspicuously, with gelatin (Fig. 3) the arginine curve lies higher than the other. With fibrin, as the data of Table VI make sufficiently evident, it is distinctly lower. These differences, it is to be supposed, depend upon characteristic differences of structure; but until we know the precise nature of the grouping determined as "arginine," it would be idle to speculate more closely upon their significance.

None of the differences observable in the behaviour of different proteins

affects the main conclusion to be derived from our experiments. This is that in the tryptic digestion of proteins the rate of liberation of arginine (or of some complex containing it) is not exceeded by that of any other single amino-acid concerning which we possess exact information. This would suggest that much of the arginine (or of the particular complex in which it is bound) is situated, as tyrosine and tryptophan have been supposed to be, in a particularly exposed position within the molecule, or that it is held by links of a particularly susceptible configuration. It might, for instance, be imagined to lie at the ends of polypeptide chains. Such an idea receives a certain amount of support from the observations of Skraup and his co-workers, who showed that with certain proteins (though not with all) deamination [Skraup, 1908] or methylation [Skraup and Krause, 1909; Skraup and Böttcher, 1910] decreased by about 90 % the yield of arginine obtainable after hydrolysis. Since both treatments affected (or were assumed to affect) only amino-groups, Skraup concluded that in such cases the amino-group of arginine is for the most part free, and that the arginine occupies an external position in the molecule. Unfortunately for this argument the results of Skraup with deaminised caseinogen find but a partial confirmation in those of Steudel and Schumann [1929] and none at all in those of Dunn and Lewis [1921] and Wiley and Lewis [1930]. It has, moreover, been shown [Kossel and Weiss, 1909, 1, 2; 1910; Dakin, 1912; Dakin and Dudley, 1913] that racemised proteins yield upon total hydrolysis only inactive arginine; and, since "it appears likely that the conditions for racemisation of an amino-acid group require the attachment of other groups to both amino- and carboxyl radicals" [Dakin, 1912], this observation makes the hypothesis of a terminal position for arginine rather uncertain. On neither side is the evidence conclusive, and the precise reason for the ready liberation of arginine by trypsin remains therefore a matter of speculation.

While the liberation of arginine by trypsin is just as rapid as that of tyrosine or tryptophan it is apparently not so complete. In all of our experiments a certain definite proportion—with fibrin one-third, with the other proteins studied one-fifth—resisted indefinitely the hydrolytic action of the enzyme. In this respect our experiments are in accord with certain earlier observations. Siegfried and Lindner [1910], for instance, after submitting caseinogen to the action of trypsin for 20 days, isolated from the digest a "peptone" which upon total hydrolysis still yielded arginine; and similar trypsin-resistant and arginine-containing peptones have been prepared from gelatin and fibrin [Siegfried, 1902, 1, 2; 1903; Müller, 1903; Krüger, 1903].

The fact that only part of the arginine of proteins is rendered by trypsin accessible to the action of arginase, suggests that arginine exists within the protein molecule in at least two different modes of combination. Both Felix [1922] and Edlbacher [1924] have already, on quite other grounds, been led to consider this possible. Simms [1928, 1, 2] has suggested that much of the arginine obtained by acid hydrolysis does not exist in the parent molecule as

such, but as a "pre-arginine" group of unknown (possibly cyclic) nature. There is, however, no quantitative correspondence between our results and the proportions of arginine and "pre-arginine" reported by Simms. In edestin, for instance, he finds evidence for the presence of "pre-arginine" only. It would seem therefore that tryptic as well as acid hydrolysis results in the transformation of "pre-arginine" into arginine. The nature of the trypsin-resistant union remains quite unknown. One might speculate on the possibility that it involves the guanidine group in arginine, the occasional participation of which in the linking of amino-acids has been thought probable by Felix [1922, 1925; Felix and Harteneck, 1927]; but the views of Felix, which are based chiefly upon a study of the peptic digestion of histone, are not supported by the work of Waldschmidt-Leitz and Künstner [1927], and the available evidence is mostly in favour of the idea that all of the guanidine groups within the protein molecule are uncombined [Kossel and Kennaway, 1911; Kossel and Cameron, 1912; Sakaguchi, 1925].

If it is true that exactly four-fifths and no more of the arginine of gelatin can be liberated by trypsin, it follows that the number of arginine groups in the molecule of gelatin is five or a multiple thereof. The same conclusion may be drawn with respect to caseinogen, edestin and egg-albumin. On the other hand, in fibrin, which appears to yield only two-thirds of its arginine to trypsin, the number of arginine groups indicated is a multiple of three. These deductions are not affected by the question whether the arginine we determined was or was not entirely free, but they do of course depend upon the correctness of our estimates of the maximum amount that trypsin can liberate. As intimated in the experimental part of the paper all of these estimates are subject to a possible revision; the calculations based upon them possess therefore for the present a merely provisional value. It may however be pointed out that conclusions consistent with our own have been reached, in an entirely different way, by Cohn, Hendry and Prentiss [1925]. Considering the composition of proteins in relation to the problem of their molecular weight, these authors are led to infer that the minimum number of arginine groups in the molecule must be for gelatin five, for egg-albumin ten, and for fibrin six. For caseinogen they give a minimum of three, corresponding to a molecular weight of 12,800; but if, as they believe, the molecular weight of caseinogen is really 192,000, the actual number of its arginine groups must be 45, a multiple not only of 3 but of 5. As for edestin, its probable molecular weight—29,000 [Cohn *et al.*, 1925]—and arginine content—26·7 % of the total nitrogen [Hunter and Dauphinee, 1930]—are consistent with the assumption that it contains 25 (5×5) arginine groups. We would not be understood as laying undue stress upon these correspondences; for it is obvious that by assuming a molecular weight sufficiently large it would be possible to harmonise almost any pair of estimates of the kind under consideration.

One point more deserves perhaps some comment. In fibrin only two-thirds of the arginine appeared to be susceptible to the action of trypsin, and even

that was liberated rather slowly. From Witte-peptone, a product derived from fibrin by peptic digestion, the arginine is liberated not only much more rapidly, but also to a greater extent. That the fraction set free should be almost exactly four-fifths may be more or less fortuitous, for Witte-peptone is of course not a single substance. The point of interest is that arginine linkages, which in the original protein are inaccessible to trypsin, are rendered accessible by a previous treatment with pepsin. One of us is now engaged in a further study of this interesting phenomenon.

SUMMARY.

1. A study has been made of the rate at which arginine (or an arginine complex susceptible to the action of arginase) is liberated during the tryptic digestion of caseinogen, gelatin, edestin, fibrin, egg-albumin and Witte-peptone.

2. In the case of the first two the initial rate of liberation is so great that half of their total potential arginine may easily be set free within 3 hours. The speed with which Witte-peptone yields its arginine is but slightly inferior. With the other proteins the appearance of free arginine is a much more gradual process. Egg-albumin exhibits a quite peculiar behaviour, in that the liberation of arginine (like that of free amino-groups in general) commences only after a latent period of one or two days.

3. In no case did trypsin liberate from a protein the total amount of arginine obtainable on complete hydrolysis. The yield with fibrin was only two-thirds of the possible maximum, with each of the others four-fifths. These fractions appear to be characteristic.

4. In the acid hydrolysis of gelatin arginine is liberated less explosively and at a more regular rate than in its tryptic digestion.

5. The possible bearing of these findings upon certain problems of protein structure is discussed.

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CXXIX. THE DETERMINATION OF BLOOD-SUGAR.

I. CRITICAL ANALYSIS OF THE REDUCTION OF ALKALINE COPPER REAGENTS BY GLUCOSE AND OTHER SUBSTANCES.

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Many and varied have been the methods employed for the determination of the sugar in the blood but so far there has been no really exact method evolved. The object of the present investigation is an attempt to evolve a method which would ultimately be satisfactory for the determination of the so-called "bound" sugar. It was first of all necessary to undertake a critical examination of the various methods in current use for the determination of the free sugar. Finally it was decided to limit this part of the work to an examination of the reduction of alkaline copper reagents by pure glucose and certain other substances found in blood.

All workers have expressed the blood-sugar in terms of glucose although at present there is much controversy as to whether the sugar of the blood exists as glucose, a mixture of glucose and other sugars, or as a more active form than the ordinary equilibrium mixture. For a method to be satisfactory the following criteria must be satisfied.

(1) Other substances present in a blood filtrate must not reduce the reagent and must not affect the reduction of the reagent by glucose.

(2) Glucose added to blood must be determined quantitatively.

The various methods so far employed to meet the first condition attempt:

(1) to precipitate as much of the nitrogenous compounds in the blood as possible. Reagents containing mercury and zinc salts have been used for this purpose [Bierry and Moquet, 1924; Harned, 1925; West, Scharles and Petersen, 1929; Somogyi, 1929];

(2) to add to the reagent to be reduced some substance or substances which will inhibit reduction so that within the time of heating only the sugar reduces the reagent [Benedict, 1927];

(3) to determine the total reducing power of a non-protein filtrate of blood, then to remove the sugar in the blood either by yeast fermentation

[Hiller, Linder and Van Slyke, 1925; Van Slyke and Hiller, 1926] or by glycolysis [Hiller, Linder and Van Slyke, 1925] and then to determine the so-called "residual" reduction value of the blood filtrate after this treatment. The difference between the total reducing value and the "residual" reduction value is assumed to represent the true blood-sugar.

All the chemicals used in this work were of A.R. standard—British Drug Houses, Ltd. The glucose was dried in an air-oven at 90° for 1 hour before being weighed out. It was found that a 1 % solution of glucose made up in saturated benzoic acid kept quite a considerable time. The various strengths of sodium thiosulphate were freshly prepared each day from a stock 0·1 *N* solution, which was tested from time to time against 0·1 *N* potassium dichromate and also 0·1 *N* potassium iodate. The keeping properties of the 0·1 *N* thiosulphate solution were found to be increased considerably by the addition of 2 g. potassium bicarbonate per litre.

Due allowance has been made for the ten-fold dilution of the constituents of blood which results from the deproteinisation according to the technique of Folin and Wu using sodium tungstate and sulphuric acid.

The Shaffer and Hartmann method.

The Shaffer and Hartmann method [1920–21] was examined from three standpoints.

(1) Using pure glucose the reduction was carried out in air as described in the original paper.

(2) The reduction was carried out in an atmosphere of nitrogen.

(3) The reagent was made up in two separate solutions (*a*) containing copper sulphate, tartaric acid and sodium carbonate, (*b*) containing potassium iodide, iodate and oxalate. The reduction was carried out with solution A, solution B being added afterwards. This reaction was studied in an atmosphere of nitrogen and also in air.

The first line of investigation was carried out as follows. In an 8 × 1 inch test-tube 10 cc. of the glucose solution and 10 cc. of Shaffer-Hartmann reagent were mixed. This was placed in a boiling water-bath for 10 minutes and then cooled to about 40°. 10 cc. of *N* H_2SO_4 were added, the whole was well shaken for about 2 minutes and then titrated with 0·0125 *N* thiosulphate solution.

For the determination under (2) the test-tube containing the mixture of glucose and alkaline copper solutions was evacuated and then filled with a slow stream of nitrogen. This was repeated several times to remove air dissolved in the liquid, until the tube and fluid contained practically only nitrogen. The determination was carried out then as under (1).

For the determination under (3) the following solutions are required:

(*a*) Copper sulphate $CuSO_4$, $5H_2O$ ... ... 5 g.
Tartaric acid ... ... 7·5 g.
Sodium carbonate (anhydrous) ... ... 40 g.
} per litre.

(*b*) Sodium carbonate ... ... 1 g.
Potassium iodide ... ... 20 g.
Potassium iodate ... ... 1·4 g.
Potassium oxalate ... ... 36·8 g.
} per litre.

(*c*) $2N$ H_2SO_4

The technique in air was as follows. In an 8×1 inch test-tube 10 cc. of glucose solution and 10 cc. of solution A were mixed and put into a boiling water-bath for 10 minutes. The tube was then cooled and 5 cc. of solution B added, followed by 5 cc. of $2N$ H_2SO_4. The whole was then well shaken and titrated with $0{\cdot}0125N$ thiosulphate solution. It will be noted that the final concentrations are exactly the same as in the original method (1). The above procedure was repeated, the tube being filled with nitrogen as described under (2).

The results are expressed in terms of cc. $0{\cdot}0125N$ thiosulphate, being the difference in titration value of a blank using 10 cc. of distilled water and a determination using 10 cc. of the known glucose solution.

Table I.

Concentration of glucose (mg. per 100 cc.)	Shaffer and Hartmann original reagent		Modified solutions	
	Air	Nitrogen	Air	Nitrogen
40	11·55	12·25	12·3	12·3
30	8·55	9·25	9·3	9·3
20	5·5	6·0	6·05	6·05
10	2·4	2·85	2·9	2·9
8	1·85	2·3	—	—
4	0·65	0·95	—	—

It is well known that glucose when heated with a solution of an alkaline carbonate is easily oxidised by the oxygen dissolved in the solution. In the Shaffer and Hartmann method the potassium salts in the reagent retard the reduction so that some of the glucose is oxidised by the dissolved oxygen before it is capable of reducing any copper. This explanation would account for the higher results obtained when the reduction is carried out in an atmosphere of nitrogen. It will be observed that when the potassium salts are not incorporated in the alkaline copper reagent but added after reduction the same results are obtained irrespective of whether the reduction is carried out in an atmosphere of air or nitrogen. This demonstrates that the amount of glucose removed by atmospheric oxygen prior to reduction of the copper is negligible under these conditions. These results also agree with the results obtained by the original Shaffer and Hartmann method carried out in an atmosphere of nitrogen.

It was decided to investigate the effects of varying the concentrations of the various items in the above alkaline copper reagent.

The amount of copper reduced in relation to the concentration of copper sulphate, tartaric acid and sodium carbonate.

Some substance must be incorporated in an alkaline copper solution which is capable of keeping the copper in solution. Benedict [1926] originally recommended a high concentration of sodium citrate, as he considered that such a reagent though not so sensitive to reduction as one containing a tartrate is nevertheless more specific for glucose. Later [1927] he dispensed with citrate and used a low concentration of tartrate, together with alanine and a high concentration of sodium nitrate. Folin and Wu [1919] and later Folin [1926] used a low concentration of tartrate—just sufficient to keep the copper in solution. These solutions containing a low concentration of tartrate are very sensitive to reduction and show very little auto-reduction when heated with distilled water in a boiling water-bath. Folin [1926] criticised Benedict's citrate reagent on the grounds that it is not sufficiently sensitive. On the other hand, Benedict criticises Folin's reagents as being too sensitive, in that they are reduced by such substances as formalin and chloroform. Folin [1929] criticises a modification of the Folin and Wu alkaline copper reagent by Somogyi and Kramer [1928] in respect of the high concentration of Rochelle salt in place of the low concentration of tartaric acid. He states that reagents having high concentrations of citrates and tartrates have high blanks thereby being unsuitable for colorimetric methods. Somogyi [1926] modified the Shaffer and Hartmann reagent by replacing part of the sodium carbonate by bicarbonate. He stated that a decrease in the alkalinity of the Shaffer and Hartmann reagent leads to an increase in the reduction—this decrease in alkalinity being obtained by replacing part of the sodium carbonate by bicarbonate. Somogyi appears to consider that alkalinity is the main factor, since two of his modifications of the Shaffer and Hartmann reagent which have been published both contain the same concentrations of carbonate and bicarbonate, but one contains 1·2 % Rochelle salt, the other 2·5 % Rochelle salt [cf. West, Scharles and Petersen, 1929]. Somogyi also uses the same concentrations of bicarbonate and carbonate in his modification of the alkaline ferricyanide reagent of Hagedorn and Jensen [1923]. It is of interest to note that in MacLean's [1916] and in the Wood and Ost alkaline copper reagents no tartrate or citrate is incorporated. Has the tartrate any effect on reduction other than simply holding the copper in solution? This was the first point to determine.

Reagents were prepared containing the same concentrations of copper sulphate and sodium carbonate but with different concentrations of tartrate. The results are shown in Tables II and III. In Table II the reagent contained:

Copper sulphate $CuSO_4, 5H_2O$ ... ... 5 g.
Sodium carbonate (anhydrous) ... ... 40 g. } per litre,

while in Table III it contained:

Copper sulphate $CuSO_4, 5H_2O$	10 g.	per litre.
Sodium carbonate (anhydrous)	60 g.	

The second point was to determine the influence of the concentration of sodium carbonate. The results are shown in Table IV. The influence of time of boiling is shown in Table V.

The amount of copper reduced was determined as follows. The reduction was carried out in a centrifuge tube. The cuprous oxide was removed by centrifuging and the supernatant fluid poured off. The cuprous oxide was then washed twice with distilled water in the centrifuge. To it was added a known amount of the Shaffer and Hartmann reagent but containing no copper sulphate, followed by the same volume of N H_2SO_4. The whole was then stirred thoroughly. The excess iodine was titrated with a standard thiosulphate solution. It will be noted that the final concentrations of all the substances are the same as in the original Shaffer and Hartmann method with the exception of the copper sulphate.

The following technique was employed in preparing the alkaline copper solutions. The requisite amounts of tartaric acid and sodium carbonate were weighed out and dissolved together in distilled water. The solution was boiled to decompose any bicarbonate which might have been formed and was then cooled. To this was added the requisite amount of copper sulphate dissolved in distilled water and the whole made up to the required volume.

Procedure. Solutions required for determining the amount of reduced copper.

1. Iodide-iodate-oxalate solution.

Sodium carbonate (anhydrous)	40 g.	per litre.
Tartaric acid	7·5 g.	
Potassium iodide	10 g.	
Potassium iodate	0·8 g.	
Potassium oxalate	18·4 g.	

2. N H_2SO_4.
3. 0·0125 N sodium thiosulphate.

In a 3×1 inch centrifuge tube 10 cc. glucose solution and 10 cc. of alkaline copper solution were mixed. The tube was then placed in a boiling water-bath for 15 minutes. It was then cooled and centrifuged. The supernatant fluid was removed and the cuprous oxide was washed twice with distilled water. 10 cc. of the iodide-iodate-oxalate solution were then added, followed by 10 cc. of N H_2SO_4. The whole was stirred to dissolve the cuprous oxide and then titrated with 0·0125 N thiosulphate. A blank was performed under similar conditions using 10 cc. of distilled water. The difference between the blank determination and a determination using glucose was taken as a measure of the amount of copper reduced by the glucose.

Table II.

Alkaline copper solutions containing:

Copper sulphate $CuSO_4, 5H_2O$	5 g.	per litre.
Tartaric acid	7·5–15 g.	
Sodium carbonate (anhydrous)	40 g.	

Concentration of tartaric acid (g. per litre)	Amount of cuprous oxide formed by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 10 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.	30 mg. glucose per 100 cc.	40 mg. glucose per 100 cc.
7·5	3·0	6·05	—	12·3
8·0	3·1	6·05	9·6	12·3
9·0	3·3	6·6	9·6	12·7
9·5	3·3	6·6	—	—
10·0	3·3	6·6	9·9	12·8
12·0	3·3	6·6	9·9	13·1
15·0	3·3	6·6	9·9	13·1

Table III.

Alkaline copper solutions containing:

Copper sulphate $CuSO_4, 5H_2O$	10 g.	per litre.
Tartaric acid	7·5–30 g.	
Sodium carbonate (anhydrous)	60 g.	

Concentration of tartaric acid (g. per litre)	Amount of cuprous oxide formed by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 2·5 mg. glucose per 100 cc.	5·0 mg. glucose per 100 cc.	10 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.	40 mg. glucose per 100 cc.
7·5	—	—	2·4	5·1	11·6
10·0	—	—	3·3	6·6	13·2
12·0	—	—	3·3	6·6	13·2
14·0	—	—	3·3	6·6	13·2
15·0	0·85	1·65	3·3	6·6	13·2
16·0	—	—	3·3	6·6	13·0
18·0	—	—	3·3	6·6	12·9
20·0	—	—	3·3	6·6	12·8
25·0	—	—	3·05	6·05	12·0
30·0	—	—	2·7	5·1	10·1

The effect of the concentrations of the sodium carbonate was then studied. The following series of solutions was prepared:

Copper sulphate $CuSO_4, 5H_2O$	10 g.	per litre.
Tartaric acid	15 g.	
Sodium carbonate (anhydrous)	30–80 g.	

Table IV.

Concentration of sodium carbonate (anhydrous) (g. per litre)	Amount of cuprous oxide formed by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 2·5 mg. glucose per 100 cc.	5 mg. glucose per 100 cc.	10 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.	30 mg. glucose per 100 cc.	40 mg. glucose per 100 cc.
30	—	1·55	—	5·75	—	—
40	—	1·65	3·3	6·6	9·9	—
60	0·85	1·65	3·3	6·6	9·9	13·2
80	0·85	1·65	3·3	6·6	9·9	13·2

The effect of time of boiling was studied, using the following alkaline copper solution:

Copper sulphate $CuSO_4, 5H_2O$	10 g.	per litre.
Tartaric acid	15 g.	
Sodium carbonate (anhydrous)	60 g.	

Table V.

Time of boiling (mins.)	Amount of cuprous oxide reduced by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate	
	5 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.
5	—	3·15
10	1·65	6·6
15	1·65	6·6
20	1·65	6·6
30	1·65	6·6

The results in Table II were checked using the following technique. Instead of centrifuging off the cuprous oxide, 5 cc. of the following solution were added:

Sodium carbonate	1 g.	per litre,
Potassium iodide	20 g.	
Potassium iodate	1·4 g.	
Potassium oxalate	36·8 g.	

followed by 5 cc. 2*N* H_2SO_4. The whole was then well shaken and titrated with 0·0125*N* thiosulphate.

The physical nature of the cuprous oxide seems to be related to the concentrations of the alkaline copper solutions. In the experiments reported in Table II the cuprous oxide was flocculent, light brown in colour and difficult to centrifuge, whereas in those of Table III with concentrations of tartaric acid above 1·2 %, the cuprous oxide was red, heavy and easily centrifuged.

Upon examining the results in Table II it will be observed that when the concentration of tartaric acid was increased above 0·75 % there was an increase of reduction up to a concentration of 0·9 %. Further increases in concentration to 1·5 % produced no further increase in reduction except in the case of the highest concentration of glucose examined. In the case of Table III the amount of reduction is also increased with increase in concentration of tartaric acid but when the concentration becomes very high, *e.g.* 2·5 % and 3·0 %, the reduction is decreased. This inhibiting effect becomes apparent in lower concentrations than these, with the highest concentration of glucose used, *i.e.* 40 mg. per 100 cc. With the reagent containing 3·0 % tartaric acid the effect of increasing the concentration of sodium carbonate was studied (Table VI).

From these results it will be seen that an increase in concentration of sodium carbonate increases the reduction to the maximum. It appears then that in high concentration tartaric acid can exert an inhibiting action which can be counterbalanced by a high concentration of sodium carbonate. It is

Table VI.

Alkaline copper solutions containing:

Copper sulphate $CuSO_4, 5H_2O$	10 g.	per litre.
Tartaric acid	30 g.	
Sodium carbonate (anhydrous)	60–100 g.	

Concentration of sodium carbonate (anhydrous) g. per litre	Amount of cuprous oxide by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate	
	10 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.
60	2·7	5·05
80	3·3	6·6
100	3·3	6·6

interesting to note, however, that, when the concentration of sodium carbonate was increased in the reagent containing 0·75 % tartaric acid in Table II, *i.e.* where the concentration of tartaric acid was insufficient to give the maximum reduction, the reduction was unchanged, even when the sodium carbonate concentration was raised to 8·0 %. The maximum reductions obtained in Tables II and III are the same even although the concentrations of copper sulphate were different, *i.e.* 0·5 and 1·0 %. In all the reagents which give a maximum reduction, the amount of reduction is unaffected by increasing either the time of heating or the concentration of sodium carbonate. The amount of reduction with these reagents is directly proportional to the concentration of glucose, with concentrations as widely different as 2·5 to 40 mg. per 100 cc. The blank determination using distilled water increases with increasing concentration of tartaric acid, but this is not a disadvantage in a non-colorimetric method. It was found that carrying out the reduction in an atmosphere of nitrogen had no effect on the results.

Determination of glucose by the estimation of the unreduced copper.

Shaffer and Hartmann [1920–21] have determined the amount of glucose by estimating the amount of unreduced copper after the reduction of an alkaline copper solution. An excess of potassium iodide and acid is added when the following reaction takes place:

$$2CuSO_4 + 4KI = Cu_2I_2 + 2K_2SO_4 + I_2.$$

The liberated iodine is titrated with standard sodium thiosulphate solution. This method was investigated by the present writer and the results were compared with those obtained by determining the amount of reduced copper directly. The following alkaline copper solution (cf. Table II) was used for this work:

Copper sulphate $CuSO_4, 5H_2O$	5 g.	per litre.
Tartaric acid	15 g.	
Sodium carbonate (anhydrous)	40 g.	

In an 8 × 1 inch test-tube 10 cc. of glucose solution and 10 cc. of alkaline copper solution were mixed and the whole was placed in a boiling water-bath for 15 minutes. It was then cooled and 10 cc. of saturated potassium iodide

solution were added, followed by 5 cc. of 25 % sulphuric acid. The whole was then well shaken and titrated with 0·0125 *N* thiosulphate. A blank determination using 10 cc. of distilled water was carried out at the same time. The difference between these two results was taken as being equivalent to the amount of copper reduced. This will be termed the indirect method, the other the direct method.

Table VII.

Concentration of glucose (mg. per 100 cc.)	Amount of copper reduced by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate	
	Direct method	Indirect method
30	9·9	9·9
20	6·6	6·65
10	3·3	3·3

The above agrees with the findings of Shaffer and Hartmann, who found perfect agreement between the two methods.

The effect of bicarbonates.

Somogyi [1926] states that a decrease of alkalinity of the Shaffer and Hartmann alkaline copper reagent leads to an increased reduction. To obtain a decrease of alkalinity Somogyi substituted sodium bicarbonate for part of the sodium carbonate. He found that as the carbonate was decreased and the bicarbonate increased the reduction increased until the concentration of sodium carbonate was 2·0 % and that of the sodium bicarbonate 2·5 %, but with a further increase of bicarbonate and decrease of carbonate the reduction began to diminish. Somogyi asserts that with the above concentrations the optimum alkalinity for reduction is obtained. Somogyi evolved a modification of the Shaffer and Hartmann reagent from these experiments.

It was decided to investigate this problem along slightly different lines. The oxidising portion of the reagent had the following composition:

Copper sulphate $CuSO_4, 5H_2O$	6·5 g.	per litre.
Rochelle salt	12 g.	
Sodium carbonate (anhydrous)	20 g.	
Sodium bicarbonate	25 g.	

In the first set of experiments the concentration of sodium carbonate was varied, keeping the concentration of sodium bicarbonate constant, and in the second set the reverse was carried out. The potassium iodide, iodate and oxalate were not incorporated in the reagent but were added as a separate solution after reduction, otherwise the original technique of the author was carried out. The amount of acid added was varied in accordance with the variation in the concentration of either the carbonate or bicarbonate. The concentration of glucose used in all experiments was 20 mg. per 100 cc.

In the first set of experiments it will be noted that an increase of concentration of carbonate has no effect on reduction. In the second set of experiments the reduction is diminished when the concentration of bicarbonate is

Table VIII.

Concentration of sodium bicarbonate 2·5 %.

Concentration of sodium carbonate (anhydrous) g. %	Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate
1·5	7·25
2·0	7·25
2·5	7·25
3·0	7·25
4·0	7·25

Table IX.

Concentration of sodium carbonate 2·0 %.

Concentration of sodium bicarbonate g. %	Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate
0	4·3
0·5	5·35
1·0	5·95
1·5	6·5
2·0	6·9
2·5	7·25
3·0	7·2
4·0	6·85

either increased or decreased from that used by Somogyi in his reagent. The solution in Table IX containing 4 % of sodium bicarbonate was taken and the effect of increasing the concentration of sodium carbonate examined (Table X).

Table X.

Concentration of sodium carbonate (anhydrous) g. %	Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate
2·0	6·85
3·0	7·1
4·0	7·25

It will be observed that by increasing the concentration of sodium carbonate, the reduction is increased to the maximum value obtained with 2·5 % sodium bicarbonate.

Bicarbonates can act in an alkaline copper reagent in the same manner as a tartrate or citrate (cf. MacLean's and the Wood-Ost reagent). In Somogyi's reagent the concentration of tartrate is insufficient to give the maximum reduction of tartrate reagents, so that by the addition of a substance of similar properties such as sodium bicarbonate, the reduction will be increased. It appears that a high concentration of sodium bicarbonate has an inhibiting action as well, this inhibiting action being neutralised by an increase in concentration of sodium carbonate. It will be noticed that similar results were obtained with solutions containing high concentrations of tartrates (cf. Tables III and VI). These experiments hardly agree with Somogyi's views. It is interesting to note that the maximum reduction obtained with tartrate-bicarbonate solutions is a little higher than with tartrate solutions.

The effect of amino-acids upon the reduction of alkaline copper tartrate reagents by glucose.

Before a method can be accepted as suitable for the determination of blood-sugar it must be shown that other substances found in a protein-free blood filtrate do not interfere with the reduction of the reagent by glucose. This was first emphasised by Holden [1926]. He showed that under certain circumstances amino-acids are capable of influencing the results considerably, not so much in reducing the reagent but by increasing the amount of reduction by glucose. Normally the blood contains 40–70 mg. of amino-acids per 100 cc. and in certain pathological conditions these figures may be exceeded. Holden investigated the method of Hagedorn and Jensen [1923] using an alkaline ferricyanide reagent and the Wood-Ost method as modified by Cole [1920]. In the latter the reagent contains copper sulphate, potassium carbonate and bicarbonate and the cuprous oxide is filtered off and washed prior to its determination. Holden examined the effects of glycine, aspartic acid, glutamic acid and cystine. With the alkaline ferricyanide reagent, the amino-acids were found to exert no influence upon its reduction by glucose. Cystine was the only one which reduced the reagent itself and the reduction obtained in the presence of glucose was found to be the sum of the reducing values when determined separately. In the case of the Wood-Ost reagent, glucose in the presence of glycine, glutamic acid and aspartic acid, caused a greater reduction than when alone. None of these amino-acids reduces the reagent itself. Glucose in the presence of cystine, which itself reduces the reagent, reduces more than when alone even when the reduction by cystine is allowed for. Holden investigated the problem for two specific reagents only, both of entirely different composition. It was decided to limit this study to the problem of the effect of these substances on the reduction of alkaline copper reagents generally.

The effect of amino-acids upon the reduction by glucose of the alkaline copper reagents in Tables II and III was examined. The centrifugal technique was employed. The concentrations of the amino-acids were a little higher than are ever present in blood. The following solutions were used:

(1)	Glycine	10 mg.	per 100 cc.	
(2)	Cystine	10 mg.	,,	
(3)	Aspartic acid	4 mg.	,,	Total 12 mg. per 100 cc.
	Glutamic acid ...	4 mg.	,,	
	Tryptophan	4 mg.	,,	

It was found that when the reagents which gave the maximum reduction were employed, the above amino-acid solutions had no effect upon the reduction by glucose. On the other hand, when those reagents which gave a lower reduction value were used, the amount of reduction by glucose in the presence of the above amino-acids was increased. Results obtained with the latter reagents of Table II are shown.

Table XI.

Concentration of tartaric acid (g. %)	Concentration of glucose (mg. per 100 cc.)	Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate	Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate in presence of amino-acids: glycine 10 mg. per 100 cc.	cystine 10 mg. per 100 cc.	aspartic acid glutamic acid tryptophan total 12 mg. per 100 cc.
0·75	10	3·0	3·3	3·3	3·3
	20	6·05	6·6	6·6	6·6
0·8	10	3·1	3·3	3·3	3·3
	20	6·05	6·6	6·6	6·6
0·85	10	3·1	3·3	3·3	3·3
	20	6·1	6·6	6·6	6·6

It will be seen that in the presence of these amino-acids the reduction is increased to the same values as are obtained with reagents giving the maximum reduction. The question therefore arises as to whether amino-acids can function in the same manner as do tartrates in alkaline copper reagents. It was decided to study the effect of substituting the tartrate entirely by an amino-acid such as glycine. Reagents of the following composition were prepared:

Copper sulphate $CuSO_4, 5H_2O$ ... ... 5 g.
Sodium carbonate (anhydrous) ... ... 40 g.
Glycine ... ... 5–10 g.
} per litre.

It was found that glycine in concentrations above 0·4 % was capable of keeping the copper in solution on boiling. The amount of reduction by pure glucose solutions was determined by the centrifugal technique as described earlier. The following results were obtained:

Table XII.

Concentration of glycine (g. %)	Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 2·5 mg. per 100 cc.	5 mg. per 100 cc.	10 mg. per 100 cc.	20 mg. per 100 cc.	30 mg. per 100 cc.	40 mg. per 100 cc.
0·6	—	—	3·3	6·6	9·9	13·2
0·7	0·85	1·65	3·3	6·6	9·9	13·2
0·8	—	—	3·3	6·6	9·9	13·2
1·0	—	—	3·3	6·6	9·9	13·2

These results show that glycine can be substituted for tartrate in an alkaline copper reagent. It will be seen that the reductions obtained with these copper-glycine solutions are the same as the maximum values obtained with alkaline copper tartrate reagents. This demonstrates why alkaline copper tartrate reagents giving the maximum reduction are unaffected by the presence of amino-acids whereas those giving lower reductions are affected. The Wood-Ost reagent as used by Holden gives lower reduction values with glucose than those in Table XII, consequently glucose would reduce more in the presence

of amino-acids, as Holden found. It is of interest to note that this reagent contains neither citrate nor tartrate but only copper sulphate, potassium carbonate and bicarbonate, so that this phenomenon is observed with alkaline copper reagents generally.

The effect of amino-acids upon the reduction by glucose of the following reagents was examined: (1) Folin and Wu [1919]; (2) Folin [1926]; (3) Shaffer and Hartmann [1920–21]; (4) Somogyi's modification [1926] of Shaffer and Hartmann. It was found that the first three reagents were reduced in greater amount by glucose in the presence of amino-acids than by glucose alone. The effect of amino-acids upon Somogyi's modification was examined in two ways: (1) the potassium salts were incorporated in the alkaline copper portion of the reagent; (2) the potassium salts were added as a separate solution after reduction. By the second method the amount of reduction by glucose is a little greater than that obtained with the alkaline copper-glycine solutions, consequently amino-acids exert no influence. In the first case the presence of potassium salts during the reduction process exerts an inhibiting action so that the reduction by glucose is much less. Amino-acids therefore tend to increase the reduction. The above deductions were confirmed experimentally.

The Folin and Wu [1919] and Folin [1926] methods are affected by the presence of amino-acids since they give a lower reduction than the maximum value for alkaline copper tartrate solutions. Their low reduction values are due to a low concentration of tartrate. The low reduction values given by the Shaffer-Hartmann method, which is also affected by the presence of amino-acids, is due to the low concentration of tartrate and to the inhibiting action of the potassium salts incorporated in the reagent. Somogyi's modification is only affected when the potassium salts are incorporated in the reagent and not added as a separate solution after reduction.

Holden [1926] states that when cystine is heated with the Wood-Ost reagent, the solution turns green with slight formation of cuprous oxide. With the reagents described in Tables II and III the solutions were turned a greenish tinge but with the concentration of cystine used (10 mg. per 100 cc.) there was no increased formation of cuprous oxide above that obtained with a blank determination using distilled water. The supernatant fluid after centrifuging contains substances which are capable of absorbing iodine. In any iodimetric method these substances would be determined as cuprous oxide, unless the cuprous oxide was removed and washed. Glycine, aspartic acid, glutamic acid and tryptophan were also found to form substances which absorb iodine, after heating their solutions with these alkaline copper reagents. These are removed in the supernatant fluid after centrifuging. These iodine-absorbing substances were found to be formed even in the cold by allowing the amino-acid solution to stand in contact with the alkaline copper reagent. This phenomenon is being investigated further. It was found that creatinine and glucose reduced the alkaline copper reagents to cuprous oxide without any formation of soluble iodine-absorbing substances.

Herbert and Groen [1929] state that when the Folin and Wu alkaline copper reagent is added to a tungstic acid filtrate and without heating the phosphomolybdic acid reagent is added, a blue colour develops. This phenomenon was not obtained with zinc or colloidal ferric hydroxide filtrates. Gulland and Peters [1930] found that if to the zinc filtrates of pigeon's blood, prepared according to the Hagedorn and Jensen technique, the alkaline ferricyanide is added and then, without heating, the potassium iodide mixture is added and the whole acidified, then upon titration with thiosulphate solution a marked absorption of iodine takes place when compared with a similar determination using distilled water.

Influence of other substances of the blood.

Hiller, Linder and Van Slyke [1925] examined the reducing powers of uric acid, creatine and creatinine in concentrations similar to those found in the blood. They found that in performing a blood-sugar determination the reduction due to these concentrations was negligible.

The present writer found that uric acid and creatinine in the following concentrations reduced the reagents.

Reduction in terms of cc. 0·0125N thiosulphate.

	10 cc. creatinine solution (10 mg. per 100 cc.)	...	1·1
	10 cc. creatinine solution (2 mg. ,,)	...	0·2
	10 cc. uric acid solution (5 mg. ,,)	...	0·05
cf.	10 cc. glucose solution (10 mg. ,,)	...	3·3

The present writer found that uric acid and creatinine in the above concentrations exerted no inhibiting influence upon the reduction of the alkaline copper reagents by glucose, the reduction obtained being the sum of their separate reduction values. Taylor [1924] states, on the other hand, that creatinine delays the precipitation of cuprous oxide in the Wood-Ost method.

It will be seen that even in pathological cases, where the uric acid and creatinine are increased, their influence will be practically negligible. It will be noted that the concentrations of uric acid and creatinine used in the above experiments were many times higher than are found in tungstic acid filtrates of blood where dilution is 1 in 10.

Holden [1926] states that urea inhibits the reduction of the Wood-Ost reagent by glucose. The effects of urea and potassium oxalate upon the reduction of the alkaline copper solutions by glucose have been examined. The following concentrations have been used: (1) urea 20 mg. per 100 cc; (2) potassium oxalate 0·1 mg. per 100 cc. These concentrations were found to exert no influence upon the reduction.

The determination of blood-sugar.

As the result of the foregoing work it was decided that the following would be the most suitable method for the determination of blood-sugar.

Solutions.

1. Alkaline copper solution:

Copper sulphate $CuSO_4, 5H_2O$	10 g.	per litre.
Tartaric acid	15 g.	
Sodium carbonate (anhydrous)	80 g.	

2. Iodide-iodate-oxalate solution:

Sodium carbonate (anhydrous)	40 g.	per litre.
Tartaric acid	7·5 g.	
Potassium iodide	10 g.	
Potassium iodate	0·7 g.	
Potassium oxalate	18·4 g.	

3. N H_2SO_4.

Method. In a 3 × 1 inch centrifuge tube 10 cc. of a protein-free blood filtrate and 10 cc. of the above alkaline copper solution are mixed. This is placed in a boiling water-bath for 15 minutes. It is then cooled and centrifuged. The supernatant fluid is removed and the cuprous oxide washed twice with distilled water. 10 cc. of the iodide-iodate-oxalate solution are added to the cuprous oxide, followed by 10 cc. N H_2SO_4. The whole is well stirred to dissolve the cuprous oxide and then titrated with 0·0125N thiosulphate. A blank determination using distilled water is carried out at the same time.

1 mg. glucose = 3·3 cc. 0·0125N thiosulphate.

Smaller volumes of blood-filtrate and smaller centrifuge tubes may be used if desired but for research purposes more accurate results would be obtained using the larger volume of filtrate. It will be noted that no specific method of preparing blood filtrates is mentioned here. This is being made the subject of a further paper.

Summary.

1. The amount of reduction of the Shaffer-Hartmann alkaline copper reagent by pure glucose is greater when carried out in an atmosphere of nitrogen than when carried out in air.

2. When the potassium salts are not incorporated in the reagent but added as a separate solution after reduction, the reduction is the same whether carried out in an atmosphere of nitrogen or air and the results are the same as obtained when the reduction of the reagent containing the potassium salts is carried out in an atmosphere of nitrogen.

3. The amount of reduction by glucose of alkaline copper solutions containing copper sulphate, tartaric acid and sodium carbonate depends on the relative concentrations of the two latter substances.

4. Bicarbonates can replace tartrates in alkaline copper solutions.

5. Glycine, aspartic acid, glutamic acid, tryptophan and cystine affect the reduction of alkaline copper reagents by glucose only under certain conditions. These have been discussed.

6. Uric acid and creatinine even in concentrations found in pathological bloods do not appreciably affect the determination of blood-sugar.

7. Urea even when present in a concentration of 200 mg. per 100 cc. blood has no influence upon blood-sugar determinations.

8. Potassium oxalate used as an anti-coagulant even in a concentration of 1 % in blood has no influence on the reduction values.

9. The composition of a modified alkaline copper reagent and a modified technique employed for the estimation of glucose in blood filtrates are described.

In conclusion I desire to thank Prof. E. P. Cathcart and Dr D. P. Cuthbertson for their very helpful criticism and advice.

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CXXX. THE DETERMINATION OF BLOOD-SUGAR.

II. THE EFFECT OF DIFFERENT DEPROTEINISING AGENTS UPON THE ESTIMATION OF BLOOD-SUGAR.

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Within recent years many modifications of the earlier methods and many new methods for the determination of blood-sugar have appeared.

De Wesselow [1919] found that the Lewis-Benedict picric acid method [1915] gave 30–50 % higher results than MacLean's method [1916]. Folin and Wu [1919] state that their method gives lower results than the Lewis-Benedict method. Höst and Hatlehol [1920], comparing four methods over a wide range, came to the conclusion that Bang's method [1918] and the Hagedorn and Jensen method [1918] compared fairly well, while that of Folin and Wu [1919] compared well with Bailey and Myers's [1916] modification of the Lewis-Benedict method. The two former gave somewhat lower results than the two latter. More recently Folin [1926] and Benedict [1927] have evolved methods by which they claim to obtain lower results than with that of Folin and Wu, but Everett [1929] has criticised Benedict's method.

Herbert and Groen [1929] found that they obtained much higher results by the Hagedorn and Jensen method when tungstic acid filtrates and not zinc filtrates were used. They also found that MacLean's method gave higher results with tungstic acid filtrates than with ferric hydroxide filtrates.

Bierry and Moquet [1924] and Harned [1925] employed mercury salts to deproteinise blood with the intention of removing substances other than glucose which were found to reduce the copper reagents used by them. These authors used the Folin and Wu alkaline copper reagent [1919] for determining the sugar in their filtrates and they claim to have obtained lower results than by the use of tungstic acid filtrates. West, Scharles and Petersen [1929] used mercury salts for deproteinisation in conjunction with a modification of the Shaffer-Hartmann reagent by Somogyi. They also state that they have obtained lower values with mercury filtrates than with tungstic acid filtrates and claim that the difference between the reducing values of the mercury and tungstic acid filtrates was equal to the non-fermentable reducing values

of the tungstic acid filtrates. Somogyi [1929, 1930] employed zinc sulphate and sodium hydroxide in the cold for preparing filtrates and claimed to obtain thereby filtrates free from uric acid, ergothioneine, glutathione and other non-fermentable reducing substances.

Stepp [1921] found that if phosphotungstic acid filtrates of blood were treated with basic lead acetate, a portion of the reducing substances was precipitated. He considered that these substances were of aldehydic nature. He found that the reducing values of solutions of pure glucose were unaffected by this treatment.

Determination of the sugar content of tungstic acid filtrates by the method recommended in Part I. (This *Journal*, p. 1161.)

All filtrates were prepared according to the technique of Folin and Wu [1919] and for accuracy 10 cc. of filtrate were used in the determinations.

As a preliminary, known quantities of pure glucose were added to determine if these could be estimated quantitatively (Table I).

Table I.

Sugar content of blood (mg. per 100 cc.)	Glucose added (mg. per 100 cc.)	Total sugar content of blood (mg. per 100 cc.)	Glucose recovered (mg. per 100 cc.)
67	0	0	0
67	50	117	50
67	100	169	102
67	200	266	199

From Table I it will be seen that, if glucose be added to blood and the mixture deproteinised with sodium tungstate and sulphuric acid, the added glucose may be estimated quantitatively.

Tungstic acid filtrates of various bloods were prepared and the reducing powers as determined by the new technique and other methods were compared. The other methods compared included: (1) Shaffer and Hartmann [1920–21]; (2) Folin and Wu [1919]; (3) Folin [1926]; (4) Hagedorn and Jensen [1923].

Zinc filtrates were not employed for the Hagedorn and Jensen method as the original method of deproteinisation was intended for non-oxalated blood. It was decided that such figures here would be unsatisfactory since all bloods used in this work were oxalated. The results given in Tables II and III were obtained and are expressed as mg. per 100 cc. blood.

From the results obtained in the previous paper it was to be expected that the new technique would give lower results than the other methods, but this does not appear to be always the case. In every case the Hagedorn and Jensen method gave a higher result and the Folin method a lower result than by the new technique. Compared with the Shaffer and Hartmann and Folin and Wu methods, the new technique at times gave higher results and sometimes lower results.

Table II.

Glucose (mg. per 100 cc.).

	New technique	Shaffer and Hartmann method	Folin and Wu method
1	105	108	99
2	103	95	91
3	102	90	89
4	112	108	100
5	74	70	67
6	86	95	95
7	102	96	105
8	100	126	89
9	87	90	89
10	86	74	89
11	108	104	115
12	83	75	87

Table III.

Glucose (mg. per 100 cc.).

	New technique	Folin method	Hagedorn and Jensen method
13	99	84	127
14	109	91	149
15	86	70	131
16	93	83	128
17	108	88	144
18	86	—	115
19	90	—	123

An excellent criterion of the amount of non-sugar substances that are being included in a blood-sugar estimation is to determine the reducing power of blood after fermentation by yeast or glycolysis. Macleod [1926] has raised serious objections to the yeast fermentation procedure in that yeast itself contains or produces during fermentation substances which reduce and that yeast may convert the non-sugar reducing substances into non-reducing forms.

It was decided to determine the residual reduction by allowing bloods to glycolyse at 37° for 24 hours with constant shaking according to the technique of Hiller, Linder and Van Slyke [1925]. At the end of 24 hours the bloods were deproteinised with sodium tungstate and sulphuric acid and the reducing values determined by the new technique. The residual reduction was found to be very low, of the order 0–6 mg. per 100 cc. blood. The residual reduction of blood as determined by the other methods is much higher, for the Folin and Wu method it is of the order 20–30 mg. per 100 cc.

Tungstic acid filtrates of blood were prepared and their reducing values determined by the new technique and by the Shaffer and Hartmann method. The remainder of the filtrates, after these determinations, was saturated with benzoic acid as preservative and allowed to stand at room temperature for a few days after which these determinations were repeated. The results given in Table IV were obtained.

It will be seen that these filtrates on standing show no appreciable change as determined by the new technique, but by the Shaffer and Hartmann method they show a marked increase. This phenomenon will be discussed later.

Table IV.

		Glucose (mg. per 100 cc.). New technique	Shaffer-Hartmann method
20		94	90
	24 hours after	93	103
21		67	50
	24 hours after	67	78
22		105	108
	48 hours after	107	122
23		74	70
	48 hours after	74	84
24		103	95
	72 hours after	105	108
25		112	108
	72 hours after	115	122

Determination of the sugar content of mercury filtrates of blood by the new technique.

Bierry and Moquet [1924] and Harned [1925] employed a solution of mercuric nitrate in dilute nitric acid but the filtrates they obtained contained high concentrations of sodium nitrate. West, Scharles and Petersen [1929] besides using this reagent employed a solution of mercuric sulphate in dilute sulphuric acid. By subsequent treatment with solid barium carbonate and metallic zinc they were able to obtain filtrates with no higher concentrations of inorganic salts than tungstic acid filtrates. A modification of this latter technique was employed to prepare filtrates for sugar determination. The method was as follows.

1 vol. of blood was laked with 10 vols. of distilled water in a flask and 1 vol. of 30 % mercuric sulphate in 10 % sulphuric acid added. The flask was stoppered and well shaken. Solid barium carbonate was then added with shaking until the reaction of the mixture was neutral to phenolphthalein. It was filtered and to the filtrate 2–3 drops of 25 % sulphuric acid were added. The filtrate was then treated with copper foil and again filtered. This filtrate, representing a 1 in 12 dilution of blood, is free from mercury and barium salts.

Solutions of pure glucose were subjected to the above treatment and it was found that their reducing values were unaffected.

Further, known quantities of pure glucose were added to samples of blood, the blood was deproteinised according to the above procedure and the reducing values of the filtrates were determined. The blood used in this experiment had been allowed to glycolyse. The results are given in Table V (mg. per 100 cc.).

Table V.

	Sugar content of blood	Glucose added	Total sugar content of blood	Glucose recovered
1	0	46	47	47
2	0	92	91	92
3	0	184	182	182

It will be seen that glucose added to blood and subjected to this treatment may be determined quantitatively.

In Table VI are given comparisons of results obtained using tungstic acid filtrates and mercury filtrates of the same bloods. The results are expressed in mg. per 100 cc.

Table VI.

	Tungstic acid filtrates	Mercury filtrates
26	90	62
27	88	60
28	90	49
29	100	62
30	141	125
31	71	44
32	85	64
33	84	51
34	79	46
35	74	51
36	83	55
37	91	73
Average	89·8	61·8

It will be seen that the reducing values of the mercury filtrates are much lower than those of the tungstic acid filtrates. The residual reductions of bloods after glycolysis and deproteinisation by this procedure were of the order 0–6 mg. per 100 cc. It may be noted that a similar figure was obtained when tungstic acid precipitation was employed. The mercury filtrates were practically free from nitrogen, approximately 6 mg. of nitrogen per 100 cc. blood being unprecipitated.

Treatment of tungstic acid filtrates of blood with mercuric sulphate.

West, Scharles and Petersen [1929] have shown that when tungstic acid filtrates are treated with mercuric sulphate and barium carbonate the reducing values of the filtrates are lowered. They state that this decrease is due to the removal of non-sugar reducing substances.

The present writer decided to repeat this. The method of procedure was as follows.

To 20–25 cc. of tungstic acid filtrate were added 1–2 drops 50 % sulphuric acid and about 0·5 g. solid mercuric sulphate. The whole was well shaken and then about 10 g. solid barium carbonate were added. After shaking, the mixture was filtered. To the filtrate 1 drop of 25 % sulphuric acid was added and then it was treated with copper foil. It was then filtered—a solution being thereby obtained free from barium and mercury salts.

The reducing values of tungstic acid filtrates were compared before and after treatment with mercuric sulphate and then portions of the untreated tungstic acid filtrates were saturated with benzoic acid (as preservative), allowed to stand at room temperature for several days and the comparisons repeated. The results, expressed as mg. per 100 cc. blood, are presented in Table VII.

Table VII.

Exp.		Tungstic acid filtrates before treatment with $HgSO_4$	Tungstic acid filtrates after treatment with $HgSO_4$
38		149	121
	3 days afterwards	150	148
39		91	75
	5 days afterwards	91	89
40		88	64
	4 days afterwards	88	88
41		76	55
	4 days afterwards	76	74
42		221	194
	5 days afterwards	221	222
43		100	85
	5 days afterwards	100	101
44		83	66
	7 days afterwards	82	80
45		160	155
	5 days afterwards	160	162
46		85	70
	6 days afterwards	85	85
47		100	82
	6 days afterwards	102	101
48		91	74
	4 days afterwards	91	91
49		115	93
	4 days afterwards	116	114

Discussion.

The reducing values of blood deproteinised with the mercuric sulphate reagent are much lower than when deproteinised with sodium tungstate and sulphuric acid. The reducing values of tungstic acid filtrates are lowered by treatment with mercuric sulphate when the treatment is carried out immediately after deproteinisation, but, when these same filtrates are allowed to stand at room temperature for several days, treatment with mercuric sulphate has no effect upon their reducing values. The reducing values of tungstic acid filtrates are unaffected by standing at room temperature when saturated with benzoic acid.

Since the reducing values of tungstic acid filtrates and mercury filtrates of glycolysed bloods are not capable of estimation, these differences observed with fresh tungstic acid filtrates and bloods cannot be due to the non-sugar fraction of the blood as stated by West, Scharles and Petersen [1929].

Martland, Hansman and Robison [1924] state that if laked blood is incubated at 37° the reducing value increases as determined by the Hagedorn and Jensen method. They attribute this to the presence of a hexosephosphoric acid which they state is present in blood in appreciable amounts. Grevenstuk [1929] states that zinc hydroxide as used in the above method precipitates degradation products of proteins incompletely. Martland, Hansman and

Robison found that the inorganic phosphorus also increased but at a much greater rate than the reducing value.

From the experimental evidence in this paper it seems that a part of the sugar in tungstic acid filtrates is in combination with one or more substances in the filtrates, this or these substances being precipitated during the mercuric sulphate treatment, the combined sugar being precipitated as well. On standing a slow hydrolysis takes place, setting free this "bound" sugar so that at this stage it would not be precipitated by the mercuric sulphate treatment.

It has been shown by glycolysis experiments that only sugar has been determined in tungstic acid filtrates by the technique described in the preceding paper. This "bound" sugar is also estimated completely by this technique since the reducing values of tungstic acid filtrates are unaltered by standing. The lower values obtained by the Folin and Wu [1919], Folin [1926] and Shaffer-Hartmann [1920–21] methods are probably due to the fact that this "bound" sugar is being incompletely determined. The increase of the reducing values of tungstic acid filtrates on standing, as determined by the Shaffer-Hartmann method, is due to the hydrolysis of the "bound" sugar.

The low reducing values of iron and zinc filtrates in the MacLean [1916] and Hagedorn and Jensen [1923] methods respectively are no doubt due to the fact that, in the course of the precipitations, the "bound" sugar is removed.

It will be noticed that bloods deproteinised with mercuric sulphate directly give much lower results for free sugar than do tungstic acid filtrates similarly treated. This is probably due to a partial hydrolysis of the "bound" sugar taking place during the initial tungstic acid precipitation and subsequent filtration.

The unknown sugar combination appears to be very loose and to break down easily.

Summary.

1. By the new technique recommended in the preceding paper the sugar content of tungstic acid filtrates is determined completely. Non-sugar substances present in the filtrates do not appreciably affect the results.

2. The sugar content of tungstic acid filtrates as determined by the Folin and Wu [1919], Folin [1926] and Shaffer-Hartmann [1920–21] methods are low.

3. Part of the sugar in a tungstic acid filtrate is in combination with other substances in the filtrate. In this form it is precipitated by treatment of the filtrate with mercuric sulphate and barium carbonate.

4. On allowing a tungstic acid filtrate to stand, however, a hydrolysis appears to take place, so that the "bound" sugar becomes free.

In conclusion I desire to thank Professor E. P. Cathcart and Dr D. P. Cuthbertson for their very helpful criticism and advice. I also wish to express my thanks to the Medical and Surgical Staffs of the Royal Infirmary, Glasgow, for their active co-operation in supplying me with samples of blood.

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CXXXI. SOME FACTORS INVOLVED IN THE EXPERIMENTAL PRODUCTION OF PELLAGRA IN RATS.

I. PRELIMINARY.

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(*Received July 2nd, 1930.*)

Owing to the work of Goldberger and his collaborators it has been shown without doubt that pellagra is a deficiency disease, curable and preventable by the administration of a pellagra-preventive factor contained, notably, in yeast. This P–P factor of Goldberger so far has not been separated from the growth-promoting component of the vitamin B complex.

That a pellagra-like condition, where in fact the nature of the dermatitis agrees remarkably closely with that occurring in human pellagra, can be produced in rats has been firmly established. But the conditions necessary for the production of pellagra in rats would appear to be less understood. Some workers have failed to produce the classical skin lesions by feeding diets apparently lacking in the pellagra-preventive factor [*e.g.* Evans and Burr, 1928] and it appears probable that, though the absence of the vitamin is the main factor in the causation of the disease, yet there may be other contributory causes, in the absence of which the disease is less likely to occur. The case against the "poor-protein" theory has been fully discussed by Aykroyd [1930] and need not be entered into here.

A diet sometimes used for producing pellagra in rats is one consisting largely of maize, which, though sufficiently rich in vitamin B_1 to prevent symptoms of polyneuritis, is poor in the pellagra-preventive factor. The shortest time taken for skin lesions to appear on this diet is about 7 weeks [Goldberger and Lillie, 1926] but the rats may remain free for much longer.

The preliminary work reported in this paper has been the outcome of an attempt to establish a routine for the experimental production of pellagra on purified synthetic diets containing caseinogen, by carefully controlling the dosage of the vitamin B complex after the rats have just been "run out" of these factors.

Experimental.

The basal diet in use in this laboratory is as follows:

Caseinogen (Glaxo, vitamin-free)	23 %
Cane sugar...	17
Rice starch	40
Palm kernel oil	15
Salt mixture (McCollum and Davis)	5

During the course of all the experiments described in this paper, cod-liver oil was given daily to each rat in 2–3 drop doses. Marmite was given in the form of an extract prepared as follows. A weighed quantity of marmite is shaken with 80–85 % alcohol. The alcohol is distilled off *in vacuo* and the fluid placed on a water-bath for about an hour. When cold it is filtered and made up to volume so that 1 cc. of the extract represents 1 g. of the original marmite.

A group of 31 rats (pure bred black and white piebalds) was placed on the above diet. Initial weights varied from 45 to 55 g. Rats nos. 1–8, and 9–15 were litter mates, and rats nos. 16–31 were mixed litters. The rats were kept on the vitamin B-free diet till the onset of polyneuritic symptoms, which occurred in 15–20 days. The animals were then divided into five groups.

Group A. Four rats received 0·75 cc. marmite daily. Growth was normal and their condition remained excellent throughout the experimental period (2 months). (Fig. 1, Curve *A*.)

Group B. Six rats received 0·5 cc. marmite daily. Growth was good, but five developed mild skin lesions on the mouth at about the 26th day of dosing. (Fig. 1, Curve *B*.)

Group C. Four rats were given 0·5 cc. marmite incorporated into the daily ration. Some food was left each day, so that the intake of vitamin was rather less than in the case of rats in group B.

Group D. Fourteen rats received 0·25 cc. marmite extract daily. Of these, nine developed severe skin lesions in 17–25 days, and five remained free. The weights of those which developed pellagra were fairly good. (Fig. 1, Curve *C*.) Those with no pellagra were not quite so good.

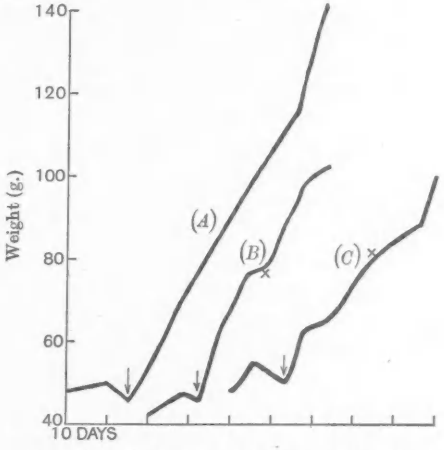

Fig. 1. Curve (*A*). Rat receiving 0·75 cc. marmite extract daily. Curve (*B*). Rat receiving 0·50 cc. marmite extract daily. Curve (*C*). Rat receiving 0·25 cc. marmite extract daily.
↓ indicates beginning of dosage. × indicates onset of skin lesions.

Group E. Three rats were given only 0·125 cc. marmite extract daily. Growth was stationary, polyneuritis becoming severe by the 28th day. The dose of marmite extract was then increased to 0·25 cc., which rapidly removed the symptoms of polyneuritis. Skin lesions then appeared after 11 more days (Fig. 2).

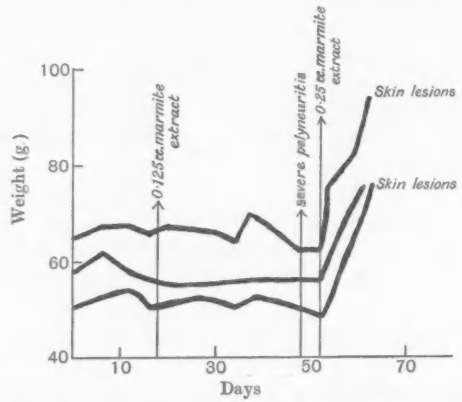

Fig. 2. Growth curves of rats receiving 0·125 cc. marmite extract daily.

From these results it is apparent that 0·5 cc. or somewhat less of marmite extract is sufficient to secure good, though somewhat irregular, growth and as little as 0·25 cc. will protect against polyneuritis for a comparatively long period, but that 0·75 cc. is required to prevent the onset of skin lesions. It should be pointed out that pellagra occurred even when growth was good. In group E, where growth was at a standstill and where polyneuritis developed, there was no pellagra, although the usual time limit for the production of skin lesions was allowed to elapse. When, however, the dose of marmite extract was increased so that growth was resumed, pellagra developed in 11 days. The mild skin lesions of the rats receiving 0·5 cc. marmite extract did not appear to get progressively worse, and after lowering the dose to 0·25 cc. the condition did not alter appreciably during the course of 3 weeks. It is to be expected that the previous higher dosage would afford some protection. Two rats in group C were rapidly cured by substituting crude marmite in equivalent doses for marmite extract. One other rat was cured by giving 0·75 cc. doses of autoclaved marmite extract, vitamin B_1 being supplied as wheat germ extract, while the fourth failed to respond to dosages of 0·5 cc. The more severe lesions occurring in group D were also cured by 0·75 cc. autoclaved marmite, but improvement was slower than that following the administration of unautoclaved marmite. 2 cc. doses of wheat germ extract alone led to no improvement.

Seasonal variation.

When an attempt was made by the method described to induce pellagra in a group of 6 rats during the months of Dec.–Feb. only two developed lesions after 10–11 weeks. The experiment was discontinued and this method of producing pellagra regarded as precarious. After good results had been obtained later in the spring however it was decided to try again during the winter months.

Group F. The experiment was carried out as described above, using 10 rats. Growth was normal in the case of those rats receiving 0·75 cc. marmite extract daily, while 0·5 cc. allowed of good growth and also appeared to afford protection from pellagra (whereas later in the year, as has been shown, slight lesions occur with this dosage). On 0·25 cc. of marmite extract growth was slow but continuous. The animals remained free from skin lesions for 3 months (as against only 3 weeks at other times in the year), but at the end of this time the typical lesions developed to a marked degree (Fig. 3, Curve *B*). No

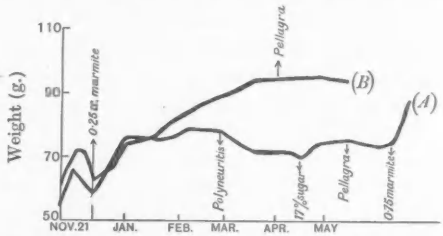

Fig. 3. (*A*). Growth curve of rat on sugar-free diet. (*B*). Growth curve of rat on 17 % cane sugar diet during winter months showing delayed onset of pellagra.

doubt the two which developed lesions in the earlier winter experiment were the forerunners of others.

Sugar-free diet.

Group G. A group of rats was placed on the following modified sugar-free diet:

Caseinogen (Glaxo, vitamin-free)	20 %
Rice starch	60
Palm kernel oil	15
Salt mixture (McCollum and Davis)	5

Here the carbohydrate content of the diet was in much the same percentage as before but consisted entirely of rice starch. The caseinogen was slightly reduced in amount. The rats were "run out" of vitamin B complex as before, and then given varying doses of marmite extract. There was a marked and surprising difference between the rats of this group receiving 0·25 cc. of the extract, and those described above. No skin lesions whatever developed but there was decided polyneuritis. The animals were "humped," had greasy fur, but were clean, whereas those with skin lesions were always very dirty.

Growth was practically stationary in the rats not receiving sugar whereas in those which developed pellagra on a sugar-containing diet growth was fairly good, even though the amount of vitamin B complex (as contained in 0·25 cc. marmite extract) was the same in both groups. After 21 weeks on the sugar-free diet the surviving rats were given the usual sugar-containing synthetic diet, the amount of marmite extract being kept as before (0·25 cc.). The polyneuritic symptoms remained much the same, but pellagra now also developed in about 11 days (Fig. 3, Curve *A*). It is therefore evident that polyneuritis and pellagra can exist together, but this point is discussed in detail later.

In view of the difference shown by the rats receiving 17 % sugar and those on the sugar-free diet, further experiments were undertaken to test the effect, if any, of giving increased amounts of sugar.

29 % sugar diet.

Group H. Four rats were fed on a 29 % sugar diet (vitamin B-free) for 21 days. They were then given 0·25 cc. marmite extract daily. The results were very similar to those obtained with rats which received the 17 % sugar diet, apart from the fact that diarrhoea was present at the commencement of the experiment. Skin lesions were present on 2 out of 4 after the 5th week of dosing. One died with no skin lesions.

57 % sugar diet.

Group I. Five rats were placed on a 57 % sugar diet (vitamin B-free) for 21 days, after which they were given 0·25 cc. marmite extract daily. After 6–7 weeks on this diet, they looked almost the same size as at the commencement of the experiment. Skin lesions appeared in 5–6 weeks after beginning dosage with marmite. When on a sugar-free diet, the ears, paws and tails of the animals looked very anaemic. Those on the 57 % sugar diet were strikingly different even before skin lesions appeared; the paws and tails being hyperaemic. This however was not so noticeable later and the weights improved somewhat. Four out of five had skin lesions.

It will be seen by the above experiments that the onset of the skin lesions was not hastened by the administration of a higher percentage of sugar to the diet, although on the 57 % sugar diet the general condition was not good. In this latter case also pellagra occurred in the absence of growth, whereas on the 17 % sugar diet pellagra occurred when growth was moderately good. These results are summarised in Table I.

Pellagra symptoms.

The question remains as to whether the skin lesions in these rats indicate true pellagra or not. A description of the symptoms shows however the closest resemblance to those described by Goldberger and Lillie [1926] and Chick and Roscoe [1927]. The front paws first became shiny and red and the nose

Table I.

Group	Carbohydrate in basal diet	Dose of vitamin B complex cc.	No. of rats fed	No. of rats with polyneuritis	No. of rats with pellagra	Days before lesions appeared	Remarks
A	40 % rice starch 17 % cane sugar	0·75	4	0	0	—	Normal growth. No lesions
B	Same as A	0·5	6	0	5	26 days	Growth good
C	Same as A	0·5 cc. mixed in daily ration of 2 rats	4	0	4	26 days	Growth good
D	Same as A	0·25	14	0	9	17–25 days	Fairly good growth
E	Same as A	0·125	3	3	3	30 days on 0·25 cc. 11 days on 0·125 cc.	Growth stationary on 0·125 cc. Resumed growth and pellagra on 0·25 cc.
F	Same as A	(*a*) 0·75	1	0	0	—	Normal growth. No lesions
		(*b*) 0·5	1	0	0	—	Good growth. No lesions
		(*c*) 0·25	6	0	5	21 weeks (Nov.-Apr.)	Subnormal growth. One death
		(*d*) 0·125	2	2	1	—	No growth on 0·125 cc. Resumed growth on 0·25 cc. One death
G	60 % rice starch No cane sugar	(*a*) 0·75	1	0	0	—	—
		(*b*) 0·5	1	0	0	—	—
		(*c*) 0·25	8	8 (3 deaths)	4/5 surviving	21 weeks on sugar-free. 11 days with sugar (Nov.-Apr.)	Subnormal growth
		(*d*) 0·125	2	2 (both died)	0	—	No growth
H	28 % rice starch 29 % cane sugar	0·25	4	0	2	35 days	Subnormal growth
I	57 % cane sugar No rice starch	0·25	5	0	4	35–40 days	No growth at first, improving later

and mouth looked sore. Later the ears became waxy or cheesy looking. The corners of the mouth became crusted and there was also an incrustation of the skin on each side of the nose and towards the eyes, giving the "butterfly" effect as described by Goldberger and Lillie. In severe cases the root of the tail gets sore and scaly. In all cases when the lesions are at their height the paws become cracked and scaly, giving the shoe and glove effect of human pellagra. The dermatitis was in all cases bilaterally symmetrical. The coats become discoloured and ill-kept, but there was no definite loss of fur.

Discussion.

The well-known seasonal incidence of human pellagra is apparently also characteristic of the rat disease. Not only are the lesions easiest to produce in the spring months, but it seems practically impossible to induce them during December, January and February. In their studies on human pellagra Goldberger, Wheeler and Sydenstricker [1920] found that no cases developed the disease during these months. The seasonal immunity of rats may account for the failure of workers when attempting to produce pellagra on diets known to be deficient in the P–P factor.

The most interesting and surprising result from the work described is the almost unfailing occurrence of skin lesions on rats receiving cane sugar as source of part of their carbohydrate when having border-line doses of vitamin B

complex. Repeated attempts in this laboratory failed to produce pellagra on diets free from sugar (though the time of year was favourable). Aykroyd and Roscoe [1929] produced pellagra in rats on a diet containing no sugar, and in other respects very similar to the sugar-free diet used during this work, but it is interesting to find that the diet was cooked for 3 hours—a process which would to some extent dextrinise the starch. It is difficult to explain the different results obtained in the presence or absence of sugar. If the sugar itself is detrimental one would expect quicker or severer symptoms when the percentage in the diet is increased, but such did not occur in the few experiments so far tried. There was however a temporary hyperaemic disorder in the case of rats receiving a 57 % level of cane sugar. The carbohydrate percentage in the sugar-free diet is 60 and this causes no disturbance whatever. The possibility of rice starch being protective must be considered. It is interesting to note that maize when used for human consumption is nearly always stated to be accompanied by either cane sugar, syrup or molasses. Blosser [1915] states that removal of cane sugar, syrup, etc., from the diet of pellagrins is in many cases sufficient to bring about complete cure. Rice-eating communities are strangely free from pellagra, but as the rice eaten by these people would be cooked, whereas that fed to rats in the present work was raw, the situation is hardly comparable from this point of view.

Kon and Watchorn [1928] showed that pellagra can be cured by transferring the rat to a diet containing a high percentage of potato starch. This is presumably due to the occurrence of refection. Refection may occur with rice starch in the food but has so far not appeared in this laboratory and was not the cause of the failure of rats on the high starch (sugar-free) diet to develop pellagra. Another very surprising result from the work described is the frequent absence of pellagra when growth has failed and polyneuritis develops later. If the B_2 factor of the vitamin B complex is identical with Goldberger's P–P factor, one would expect a failure of growth to be a constant accompaniment of the onset of pellagra symptoms, but this was not the case, as will be seen by Fig. 1, Curve *B*.

The rats which developed pellagra on the daily dose of 0·25 cc. marmite extract were practically all growing. Growth of course was far from optimal but it never quite ceased. On the other hand, cessation of growth was almost invariably accompanied by lack of pellagra symptoms (except in the case of those receiving all their carbohydrate as sugar). This was very strikingly confirmed by reducing the amount of marmite extract to 0·125 cc. daily. If pellagra occurs in 17–25 days in a large majority of cases where the daily dose of marmite is 0·25 cc. one would expect at least equally rapidly occurring lesions on the smaller dose. It was surprising to find that such was not the case. On a diet entirely free from the vitamin B complex, no pellagra appears for the simple reason that the animals succumb to polyneuritis before the lapse of the time interval necessary for the occurrence of pellagra. In the case of rats on 0·125 cc. marmite extract some severe cases of polyneuritis

developed and the animals died. But it was unexpected to find that, though some of the rats had such mild polyneuritis that they lived for a month, their weight being stationary or declining, not the least sign of pellagrous skin lesions was ever noticed, although this interval has been shown to be sufficiently long for the appearance of the first signs of pellagra. In the case of those animals on the sugar-free diet survival with polyneuritic symptoms was indeed much longer—as long as 21 weeks having elapsed with no symptoms of pellagrous skin lesions. Again, it must be emphasised that the time of year was favourable for the appearance of pellagra, this being illustrated by a group of rats on a cane sugar diet at the same time, the majority of which developed skin lesions. Further, when the group of rats on sugar-free diet was placed on a sugar-containing diet they rapidly developed pellagra.

It is possible that those rats receiving the higher dose of marmite extract (0·25 cc. daily) which did not gain weight were also suffering from mild polyneuritis, as this dose is obviously on the border line of that necessary to prevent polyneuritic symptoms with certainty. The idea that growth itself is a contributory factor to the development of pellagra is hardly tenable nor would the suggestion that the growth-promoting factor of the vitamin B complex and the P–P factor are not identical explain the non-occurrence of pellagra in the rats receiving the smaller dose of marmite. The possibility is left that the actual occurrence of polyneuritis is inhibitory to the development of pellagra, though, as we have seen, if the animals can be kept alive for a sufficient length of time (*e.g.* 21 weeks in the case of those on the sugar-free diet) it is possible to induce pellagrous symptoms in the presence of polyneuritis, thus showing that the condition of polyneuritis does not necessarily prevent pellagra. If the suggestion should prove permissible that beriberi inhibits to some extent the development of pellagra it may explain the infrequency of the latter disease amongst rice-eating communities where beriberi is rife.

Summary.

1. On a synthetic diet containing both rice starch and cane sugar pellagra could be induced in 17–25 days if the rats were first "run out" of the vitamin B complex and then given a minimum daily dose of marmite, sufficient to prevent polyneuritis.

2. In the absence of cane sugar from the diet pellagra did not appear, but all the rats developed polyneuritis.

3. Increased amounts of cane sugar in the diet did not increase the severity of the symptoms.

4. Rats suffering from polyneuritis developed pellagra after the marmite dose was raised sufficiently to protect them from polyneuritis, and in the case of those on a sugar-free diet, when sugar was added.

5. Rats were shown to have a seasonal immunity from pellagra comparable to that occurring in the human disease.

I wish to express my thanks and deep indebtedness to Sir F. G. Hopkins under whose supervision and licence these experiments were carried out, and for the use of all materials incurred.

I also thank Dr E. Watchorn for her great interest in this work.

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CXXXII. COMPARISON OF THE EFFECTS OF ARGININE AND THYROXINE UPON TUMOUR GROWTH-RATE IN THE MOUSE.

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(*Received July 7th, 1930.*)

The growth rate of a tissue may be considered to depend in part upon the amount and nature of the substances available for its nutrition. If it is found that a specific chemical substance accelerates tissue growth it should be possible to reverse this effect provided that the concentration of such a substance in circulation could be reduced.

In the case of tumour tissue there is experimental evidence that arginine induces an increase in the rate of growth [Gilroy, 1930], and it is probable that this amino-acid is necessary for the nutrition of any tissue in which cell reproduction is proceeding. It has been shown [Crabtree, 1928] that the glycolytic activity of tumours is not a specific attribute of malignant tissue, other pathological overgrowths exhibiting the same type of carbohydrate metabolism in varying degree. Since normal tissues may temporarily assume the characters of neoplastic tissues in this respect it might be assumed that they would show coincident similarities in the metabolism of protein.

Assuming therefore that a certain level of arginine concentration is necessary for the adequate nutrition of tumour tissue, and that other tissues may under special circumstances demand the same material, for reproduction or repair, it was evident that one method of reducing the amount of arginine available for the tumour would be to divert it by creating such a demand.

It is known that administration of the active principle of the thyroid to healthy subjects causes an increased excretion of nitrogen, chiefly in the form of urea [Rohde and Stockholme, 1919] and that unless the protein content of the diet is high more nitrogen is excreted than ingested, indicating that tissue protein is being destroyed [Cushny, 1924]. In view of these facts it seemed reasonable to postulate an increased catabolism of protein under the influence of thyroxine, which might affect the tumour either directly, or by stimulating metabolism in general to an extent which brought the whole body into competition for such substances as were necessary for growth and tissue repair. Such an hypothesis would also accommodate an observation (Gilroy, unpublished) that the rate of tumour growth in the pregnant mouse is exceedingly slow; under such conditions embryonic tissue is present requiring nourishment

similar to that demanded by the tumour, and the metabolism of the body as a whole is slightly increased as a result of the pregnant condition.

Experiments were therefore undertaken to test the effect of non-toxic doses of thyroxine alone and in combination with arginine. The technique and material were similar to those used in a previous investigation [Gilroy, 1930]. The calipers were specially made for the purpose of obtaining accurate measurements, with a screw adjustment (40 threads to the inch)[1].

Exp. 1. 30 mice implanted 10 days previously were divided into 3 groups. Group I received 0·3 cc. of a 10 % solution of arginine every second day. Group II were given similar treatment, but in addition were injected daily with 0·1 cc. of a 1/1000 solution of thyroxine[2], the arginine being given in the morning and the thyroxine at night. Group III were untreated controls. Caliper measurements were made on the first and last days of the experiment, 10 and 22 days respectively after implantation, and cross-sectional surface area was determined in mm.^{2}; the mice were also weighed on the same dates.

This preliminary experiment gave the following result:

Group I. Arginine:

Mean size of tumours, day	10	14·6 mm.^{2}
,, ,, ,,	22	308·2 mm.^{2}
Mean weight of mice, ,,	10	21·6 g.
,, ,, ,,	22	24·5 g.

Group II. Arginine + thyroxine:

Mean size of tumours, day	10	23·5 mm.^{2}
,, ,, ,,	22	127·4 mm.^{2}
Mean weight of mice, ,,	10	20·9 g.
,, ,, ,,	22	24·1 g.

Group III. Untreated controls:

Mean size of tumours, day	10	13·6 mm.^{2}
,, ,, ,,	22	204·8 mm.^{2}
Mean weight of mice, ,,	10	22·5 g.
,, ,, ,,	22	25·5 g.

The ratio of size on the 10th and 22nd days respectively was:

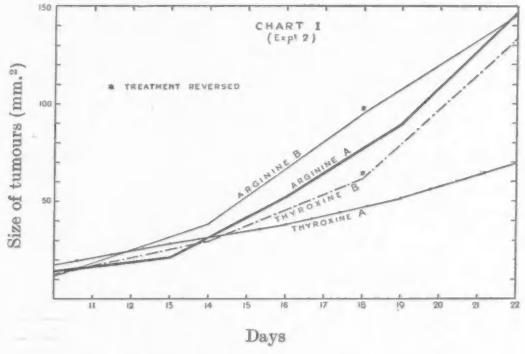
Arginine	1 : 21·1
Arginine + thyroxine	1 : 5·3
Untreated controls	1 : 15·0

Taking the ratios as an index of the rate of growth it will be seen that arginine induced a more rapid rate of growth than occurred in the untreated control group, but that the latter again exceeded that of the group receiving thyroxine. In view of the small number of animals used in this initial experi-

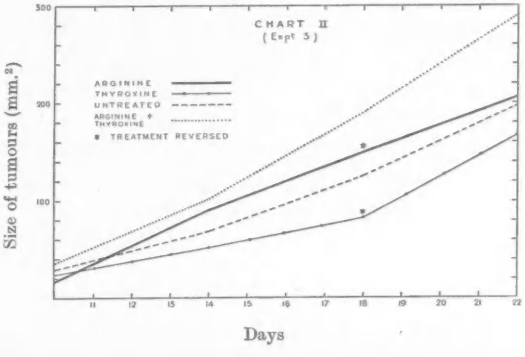
[1] Made by Messrs A. W. Hall and Son, Engineers, Edinburgh.
[2] Schering.

ment no reliance could be placed upon the results, but they were considered sufficiently encouraging to warrant further investigation upon a more adequate scale. In the succeeding experiments it was found that if arginine was given daily in conjunction with thyroxine the retarding effect of the latter was less marked (*e.g.* Exp. 4). The weights of the mice on the last day include the tumour, but it will be seen that in the arginine-treated group where the tumours were largest the increase in weight during the experimental period was no greater than in the group receiving thyroxine.

Exp. 2. 44 mice implanted 10 days previously were divided into two sections A and B, the sections being implanted from two different tumours. In each section one group was injected with arginine and the other with thyroxine but in section B treatment was reversed 18 days after implantation. The results are shown in Chart I.



Exp. 3. 46 mice implanted 10 days previously were divided into 4 groups, I receiving arginine, II thyroxine, III arginine a.m. and thyroxine p.m.; group IV were untreated controls. Treatment was reversed on the 18th day; the results are shown in Chart II.



Exp. 4. 45 mice implanted 10 days previously were divided into groups and treated as follows:

Group I. Arginine (A) 6 mice. Treatment reversed 18th day.
(B) 6 mice. Treatment continued 18th day.

Group II. Thyroxine (A) 6 mice. Treatment reversed 18th day.
(B) 6 mice. Treatment continued 18th day.

Group III. (A) arginine injected daily (6 mice).
(B) arginine injected every 2nd day (6 mice).

Group IV. Untreated controls (9 mice).

The result of this experiment is shown in Charts III, IV and V in which the first (*i.e.* treated) groups are shown separately for comparison with group IV (untreated controls).

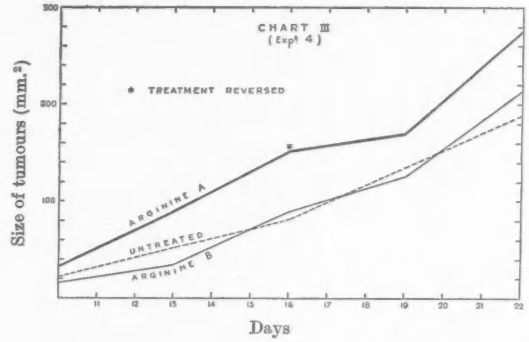
A summary of the protocols of Exps. 2, 3 and 4 is presented in the following table:

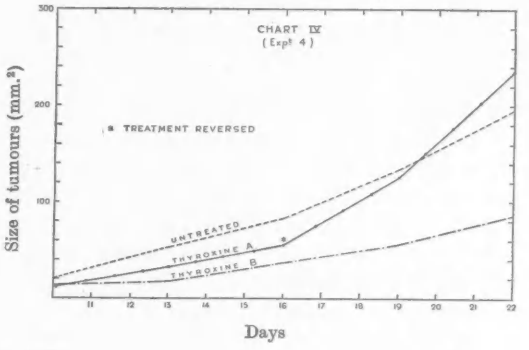
Summary of protocols.

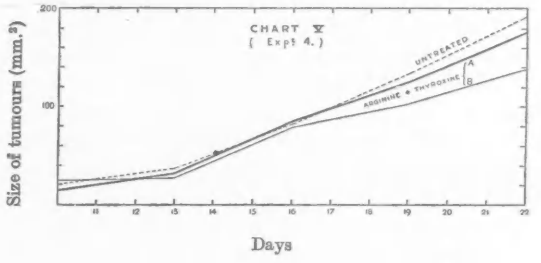
		Mean size of tumours		Mean weight of mice	
Exp.	Treatment received	Day 10 (mm.²)	Day 22 (mm.²)	Day 10 (g.)	Day 22 (g.)
II	Arginine (A)	14·5	146·5	24·3	28·3
	Thyroxine (A)	17·2	69·5	24·0	25·0
	*Arginine (B)	11·5	146·0	22·4	25·2
	*Thyroxine (B)	12·0	132·1	22·3	24·8
III	*Arginine	18·5	209·0	21·4	24·3
	Arginine + thyroxine	37·5	295·5	22·0	26·5
	*Thyroxine	26·8	169·8	22·6	25·1
	Untreated	30·7	202·3	20·4	22·8
IV	*Arginine (A)	33·8	276·6	22·1	23·6
	Arginine (B)	15·6	215·1	19·6	20·2
	*Thyroxine (A)	12·0	235·0	19·8	22·9
	Thyroxine (B)	12·5	85·5	19·0	20·4
	Arginine + thyroxine (A)	16·3	175·3	21·5	21·7
	Arginine + thyroxine (B)	26·8	139·4	20·8	22·0
	Untreated	20·7	193·4	19·7	21·7

* Treatment reversed during the 12-day experimental period.

In Exps. 2 and 3 reversal of treatment 18 days after implantation was followed by a deflection of the curve in the corresponding groups. As only one reading was possible after reversal it was doubtful whether the alteration would be maintained; in Exp. 4 an attempt was made to settle this point and the results show that the administration of arginine to a group previously receiving thyroxine caused a permanent increase in growth, and that if thyroxine was subsequently given to a group previously injected with arginine a temporary arrest of growth occurred but it was not maintained. If arginine was given daily in conjunction with thyroxine the rate of growth hardly differed from that of the controls; if given every second day the growth was correspondingly less.







Discussion.

The results of these experiments support the hypothesis that arginine and thyroxine are mutually antagonistic in respect to tumour growth, the former being an important nutritional factor which is destroyed together with other tissue proteins in the augmented catabolism induced by the action of thyroxine.

The evidence obtained that the dose of arginine can be adjusted to balance the inhibitory effect of thyroxine is an indication that protein catabolism is

primarily involved, but another aspect which should be considered is the regulation of carbohydrate metabolism by the thyroid, hypo-activity having been shown to cause significant alteration [Wells, 1925], not only in sugar utilisation, but in all oxidative processes throughout the body. There is however no evidence that a reduction in blood-sugar diminishes the rate of tumour growth, indeed according to Bouin [1927] malignant tar tumours grew with great rapidity in rabbits receiving two rabbit units of insulin daily for six months, and the animals died 4 weeks after treatment was stopped. It may be contended that the greater malignancy of such tumours renders them useless for comparison with the tumour M63, but there is also experimental evidence that insulin has no inhibitory effect upon the growth of tumour grafts in mice; Cioffari and Piccaluga [1926] found that unless insulin was given in excessive doses (1/5 of a clinical unit daily) it had no effect; in some cases death occurred in 15 days and they concluded that hypoglycaemia alone does not materially affect the rate of tumour growth; the same workers report that a carbohydrate-rich diet is without effect in accelerating the rate of tumour growth. It is quite possible that although a reduction in blood-sugar is without effect a loss of balance between the catabolic and synthetic phase due to impaired oxidative processes would retard tumour growth [Warburg, 1926], but it is questionable whether it is actually anoxaemia which has been the cause of retarded growth in experiments on reduced oxygen tension in view of the compensatory mechanism which comes into play under such circumstances [Barcroft, 1925], although the extent of acclimatisation depends upon many factors and is not rapidly attained.

Before any general significance can be claimed for these results it is necessary to consider the reaction of the mouse towards thyroxine and the relation between this neoplasm (M63), and its host. Resistance of the mouse towards thyroxine has been recorded by Abderhalden and Wertheimer [1928], and it may be argued that the behaviour of a spontaneous neoplasm towards thyroxine would be in no way analogous to that of a tumour which was essentially a graft. If mice are relatively insusceptible to thyroxine this may explain the absence of any loss of weight during the experimental period, and indicate that the same degree of hyperthyroidism would not be tolerated by other mammals without the appearance of toxic symptoms. That thyroxine might cause resorption of a graft and therefore reduce the growth of this tumour appears a valid argument, but it must be remembered that treatment was never instituted until the graft was well established, 10 days after implantation, and that actual disappearance of the graft did not occur, although the treatment materially affected its subsequent rate of growth. Tumours which had already attained a large size, such as those previously treated with arginine (*e.g.* Exp. 4) continued to grow under treatment with thyroxine, a temporary cessation being followed by a sharp rise in the curve of growth. In view of the fact that treatment was limited to a period of 12 days the full results which might follow the reversal of treatment could not be attained,

since termination of the experiment is necessitated by ulceration of tumours in one of the more rapidly growing groups.

SUMMARY.

In a series of experiments using 165 mice it was found that injections of thyroxine retarded the rate of tumour growth as compared with untreated controls; this was not accompanied by any loss of body weight during a 12-day period of treatment. Substitution of arginine for thyroxine caused a renewal of rapid growth, but if arginine had been given previously the substitution of thyroxine did not cause a permanent deflection of the curve. If both were given to the same animal the rate of growth was similar to that of untreated controls if arginine was given daily, but if given less often the effect of thyroxine predominated, indicating an antagonism in the effect of these two substances in respect to tumour growth.

The expenses of this work were defrayed by a grant from the Medical Research Council, to whom I would express my indebtedness, and I wish also to thank Professor Crew not only for scientific hospitality but for helpful criticism and advice.

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CXXXIII. A NEW METHOD FOR THE SEPARATION OF THE PRODUCTS OF PROTEIN HYDROLYSIS.

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It has long been acknowledged that the methods available for the quantitative separation of the products of hydrolysis of proteins are not entirely satisfactory. The yields of individual amino-acids are often low, and the manipulative processes involved in their separation are difficult. The well-known process, due to Emil Fischer, in which the amino-acids are separated mainly by the fractionation of their esters at very low pressure remains the standard method of separation except in some special cases. Alternative methods have been put forward from time to time, but these have been concerned with particular fractions of the hydrolysis products, and no comprehensive scheme avoiding the use of ester-fractionation has been advanced for the separation of the amino-acids. Among the newer methods of dealing with the mixture of amino-acids may be mentioned the butyl alcohol extraction method of Dakin [1920] and the calcium salt and lead salt methods of Foreman [1919]. The object of all these has been to overcome, in regard to particular details, some of the objections to the ester method of Fischer, but none has entirely dispensed with the use of the esters at some stage of the process.

The older methods enabled the early workers to investigate the hydrolysis products of many proteins with considerable success, but in no case was the complete analysis of a protein possible. A separation which accounted for 75 % of the total nitrogen represented a careful and successful fractionation; more usually little more than 60 % of the nitrogen could be traced. In some of the later hydrolyses about 85 % of the nitrogen was accounted for [Foreman, 1919].

It is evident from the results quoted that a more satisfactory technique was required.

With these considerations in view, much work has been carried out during the past 7 years, under the direction of the late Professor S. B. Schryver, with the object of finding a method for the separation of the amino-acids from among the hydrolysis products of proteins which should fulfil, as far as possible, the following conditions:

(i) the method should be capable of furnishing a maximum yield of pure individual amino-acids;

(ii) the use of the esters should be avoided entirely;

(iii) the method should be as simple as possible.

A technique has been evolved which, although not yet perfect, more nearly fulfils these conditions than any previously available.

The method is due to the late Professor Schryver, who was assisted by a number of workers, including the author, who carried through the hydrolysis of zein described in this paper; this constituting the first example of a complete analysis carried out by the method.

An outline of the method has already been published from this laboratory by Town [1928] to whom much of the technique for separating the amino-acids is due.

The separation is based on the fact that the different amino-acids, and certain of their derivatives, in particular their copper salts, show widely different degrees of solubility in solvents such as water, methyl alcohol and ethyl alcohol. The success of the method depends upon the use of very pure dry solvents and thoroughly dry copper salts.

In drying the copper salts, the only satisfactory drying agent was found to be "absolute" acetone. By its use the copper salts are obtained in the form of a fine dry powder, which readily responds to fractionation by means of the appropriate solvents.

The main fractions so obtained are the following.

A. *Copper salts insoluble in water.*

This fraction contains leucine and phenylalanine together with aspartic acid.

B. *Copper salts soluble in water.*

These salts are further separated by extraction with methyl alcohol, giving:

(i) *Copper salts insoluble in methyl alcohol.*
Alanine, tyrosine, glutamic acid, histidine and arginine (also glycine and bases if present).

(ii) *Copper salts soluble in methyl alcohol.*
Valine, hydroxyvaline, proline and prolylphenylalanine.

The further separation of these amino-acids is described in detail below.

The use of the new technique has made it possible, in the case of zein, to isolate 87·9 % of the nitrogen from the neutralised and filtered hydrolysate (which contains only 87·2 % of the total N of the zein) as amino-acids in the approximately pure form, and, by examination of all the precipitates obtained at various stages, a total of 93·5 % of this nitrogen has been traced.

The analysis by this method has not only accounted for a higher total of nitrogen than earlier analyses, but it has in addition led to the discovery in zein of two constituents hitherto undetected—namely hydroxyvaline and prolylphenylalanine.

Experimental.

Preparation of zein.

The zein used for hydrolysis was prepared from maize flour by repeated extraction with 73 % alcohol; about 10 extractions were made, the maize being left each time to stand overnight under the alcohol. As 73 % alcohol extracts the colouring matter from maize as well as zein, this may be used as a fair indication of the completion of the extraction. The alcohol-soluble portion was dried, and the colouring matter removed by repeatedly extracting with dry acetone. This not only dissolves the colouring matter, but also absorbs the remaining moisture from the zein, which thus becomes granulated to a fine powder. A 3·4 % yield of zein was obtained by this method from the sample of maize used.

Hydrolysis of zein.

The zein so prepared contained 7·2 % water and 0·3 % ash.

250 g. of this material, equivalent to 231 g. moisture-free and ash-free zein (containing 17·5 % nitrogen) were hydrolysed by boiling with 4 times the bulk of 25 % sulphuric acid under a reflux condenser for 24 hours.

On cooling and filtering the hydrolysis solution, a considerable amount of material was found to be insoluble, 18·8 g. in all, equivalent to 8·1 % by weight of zein. This substance contained 9·5 % of nitrogen, but on extraction with ether and alcohol a large quantity of fat was removed, the remaining material being found to contain 12·1 % nitrogen. It appears to be, most probably, incompletely hydrolysed protein protected from hydrolysis by fat; after removal of the fat this material can be hydrolysed in the usual way.

After filtering off this insoluble substance, the filtrate was neutralised with baryta; the precipitate of barium sulphate was boiled up with water 7 or 8 times to extract as much as possible of the amino-acid solution, as this is where the greatest loss of nitrogen takes place; it was found to be more efficient to dry the precipitate in the oven between each washing and grind it to a powder before extracting with boiling water.

An estimation of the nitrogen in this barium sulphate after washing showed that 3·42 g. had been retained. A large part of this is most probably the humin-nitrogen.

After removal of barium sulphate, the nitrogen of the total filtrate, together with the washings, was found to be 35·33 g. This figure is used throughout this paper as the basis on which the percentages of amino-acids are calculated.

The nitrogen at this stage is accounted for as follows:

Table I.

N in insoluble material	1·78 g.
N in barium sulphate precipitate	3·42 g.
N in neutralised hydrolysate	35·33 g.
Total	40·53 g.

This table shows the total of 40·53 g. of N from 231 g. protein, equivalent to 17·5 % of N in zein. This confirms the figure obtained for the N content of the ash-free zein.

Removal of ammonia.

An estimation of the ammonia present (Fraction C, Table II) was made on an aliquot part of the hydrolysis solution; this gave an equivalent of 7·40 g. N as ammonia, *i.e.* 20·95 % of the total. The ammonia from the whole filtrate was removed by making it just alkaline with baryta and drawing air through for several hours with slight warming of the solution.

Conversion to copper salts.

When no further ammonia could be detected the solution was made quantitatively free from barium and sulphuric acid; it was then converted into the copper salts by adding copper carbonate to the boiling solution until there was no further effervescence; after boiling for a short time it was evaporated to dryness with excess of the carbonate; the solid so obtained was extracted with cold water in the mechanical shaker for 1 hour, and the extract again evaporated to dryness with more copper carbonate. This was repeated two or three times to ensure complete formation of the copper salts.

This process gives the first stage of separation into two fractions: copper salts soluble in water, and copper salts insoluble in water.

A. *Copper salts insoluble in water.*

This fraction contained the excess of copper carbonate together with the copper salts of leucine, phenylalanine and aspartic acid. An advantage of the copper-salt method is that the two dicarboxylic acids (glutamic and aspartic acids) appear in different fractions.

The whole material was dissolved in dilute sulphuric acid and the copper removed by hydrogen sulphide; the solution was made alkaline with baryta, the barium sulphate was filtered off, and the barium aspartate precipitated by adding 3 volumes of 95 % alcohol. For this precipitation the concentration was approximately 8 g. N in a litre of the solution, to which 3 litres of alcohol were added (Foreman's [1914] method modified by Dakin [1920]).

The barium aspartate was decomposed by adding sulphuric acid quantitatively, the free acid was evaporated to a syrup and granulated with absolute alcohol.

6·55 g. of crude aspartic acid were obtained containing 10·41 % N; this is therefore approximately pure aspartic acid even before recrystallisation. 0·68 g. N was thus isolated as aspartic acid, *i.e.* 1·92 % of the total N in the $BaSO_4$ filtrate from the hydrolysate.

From the crude amino-acid a sample was prepared by boiling with charcoal and repeated recrystallisation, which gave 10·50 % N (calculated 10·53 %).

The alcoholic filtrate from the barium aspartate was freed from excess baryta by sulphuric acid, the barium sulphate being removed by filtration, and the alcohol distilled off; when neutral the solution was concentrated on a water-bath until leucine began to crystallise out. On cooling, large quantities of very nearly pure leucine (containing 10·61 % N) separated, more crystals being obtained on concentrating further. The remaining solution containing the phenylalanine and the rest of the leucine was converted into the zinc salts. After drying at 110° the zinc salt of leucine becomes quite insoluble in cold water, whereas that of phenylalanine is readily soluble. For the preparation of the zinc salts the solution was made up roughly to 500 cc. (containing approximately 5 g. N); excess of freshly precipitated zinc hydroxide was added to the boiling solution, the whole being kept boiling for half an hour; the precipitate was filtered off and the filtrate again boiled with fresh zinc hydroxide to ensure complete formation of the zinc salts. The filtrate was evaporated to dryness and dried at 110°. The precipitate was also dried at this temperature, and each was then extracted with cold water. The extract was evaporated down and again dried at 110° and extracted with cold water; this was repeated until all redissolved.

The insoluble zinc salt of leucine was decomposed in alkaline solution with hydrogen sulphide and evaporated to dryness. The total weight of crude leucine obtained by direct crystallisation and by decomposition of the zinc salt was 51·43 g., containing in all 5·31 g. N, equivalent to 15·05 % of the total N.

From this a specimen of pure leucine was prepared by recrystallisation from hot water, which contained 10·68 % N (calculated 10·69 %).

The zinc salt of phenylalanine was similarly decomposed, and on evaporation the solution of the free acid gave 15·18 g. of crude phenylalanine containing 8·05 % N, *i.e.* 1·28 g. N in all or 3·62 % of the total N.

On repeated recrystallisation a sample was prepared giving 8·37 % N (calculated 8·49 %).

The phenylalanine was found to be much more difficult to obtain in the pure form than leucine, having a tendency to deliquescence.

B. *Copper salts soluble in water.*

These were evaporated as nearly to dryness as possible, and then granulated by stirring the syrup with dry acetone to absorb the last traces of moisture. The acetone is poured off through a filter, and a further batch added to the copper salts; this is repeated many times until all the moisture has been removed and the copper salts appear as a granular powder, with no trace of stickiness.

The last traces of acetone are removed by drying in a vacuum desiccator, and finally at 110° for half an hour.

The copper salts, when dry, were extracted repeatedly (about 10 times) with dry methyl alcohol in the mechanical shaker, until no more dissolved. (The methyl alcohol extract never becomes quite colourless as some moisture

is absorbed from the air, but when three consecutive extractions gave the same depth of blue, the process was judged to be complete.)

In this way two further fractions were obtained, copper salts soluble, and copper salts insoluble, in methyl alcohol.

B1. *Copper salts insoluble in methyl alcohol.*

This fraction contained the copper salts of alanine, glutamic acid, tyrosine, arginine and histidine (glycine, if present, occurs in this fraction, also bases).

After confirming that all would redissolve in water, the copper was removed by hydrogen sulphide, and the solution evaporated down to about 1500 cc. (containing approximately 11 g. N). It was then made alkaline with baryta, the barium sulphate was filtered off, and on addition of three times the volume of 95 % alcohol barium glutamate was precipitated. (Foreman's method, as used in Fraction A for aspartic acid.)

By carrying the precipitation through twice, a clean granular precipitate of barium glutamate was obtained. This precipitate was quantitatively decomposed with sulphuric acid, the barium sulphate filtered off, and the free acid evaporated to dryness; large quantities of crude glutamic acid crystallised out during this evaporation, and were filtered off, the residue being granulated with alcohol. Before recrystallisation this material contained 9·3 % N (calculated 9·52 %).

The total yield of crude glutamic acid weighed 73 g. and contained 6·78 g. N, equivalent to 19·19 % of the whole.

On boiling up with charcoal and recrystallising from water glutamic acid was prepared containing 9·50 % N.

After the removal of the glutamic acid, the barium salts soluble in alcohol were quantitatively decomposed with sulphuric acid, the barium sulphate was filtered off and the solution of free acids concentrated to boil off the alcohol. On further concentration to a small bulk, crystals of tyrosine separated.

These, without recrystallisation, contained 7·73 % N (calculated 7·74 %) and weighed 5·04 g. containing therefore 0·39 g. N, *i.e.* 1·14 % of the total.

The next stage was the separation of the bases, histidine being first removed by formation of its zinc salt and precipitation with mercuric chloride (Schryver's method).

The filtrate from the tyrosine was converted into the zinc salts by boiling for half-an-hour with excess of freshly precipitated zinc hydroxide, after which the excess hydroxide was filtered off, and a solution of mercuric chloride added carefully until there was no further precipitate. Histidine forms a complex compound which is insoluble in water. This was filtered off and decomposed in acid solution with hydrogen sulphide to precipitate the mercury; the solution was filtered and the chlorine removed by adding silver sulphate; the silver was precipitated by hydrogen sulphide, filtered off, and the filtrate made alkaline with baryta; the zinc was then removed by hydrogen sulphide, the

barium sulphate and zinc sulphide being filtered off together. The filtrate when neutral was concentrated on a water-bath, but did not crystallise; it contained 0·70 g. N, equivalent to 1·98 % as histidine. The solution was evaporated to a thick syrup and granulated with alcohol; this gave a fine white powder. As, however, this gave a nitrogen content low for histidine, it was redissolved, boiled with charcoal and again granulated with alcohol. The nitrogen value was still low—24·86 % (calculated 26·96 %) and a trace of ash was found on incineration. The experiments of other workers in this laboratory indicate that histidine alone is precipitated by this method, which suggests that the material prepared from zein was histidine contaminated with inorganic matter probably brought down from the protein itself. No trace of mercury, zinc, silver or barium could be detected in the ash. Further research is necessary on the histidine separation and is in progress.

The remaining solution containing alanine and arginine was freed from chlorine by silver sulphate, and from mercury, silver and zinc by hydrogen sulphide. The solution was concentrated to about 500 cc. (containing 2·5 g. N) and made 0·5 % acid with sulphuric acid; the arginine was then precipitated as the flavianate by Kossel's and Staudt's method [1926].

A solution of flavianic acid in cold 0·5 % sulphuric acid was added and the whole well shaken. (About 2 g. of flavianic acid are required for every g. of arginine present.)

After standing for 3 days a crystalline precipitate formed, which was filtered off and washed with 0·5 % sulphuric acid. These crystals contained no inorganic matter, and charred at 248°; they were still unmelted at 280°. They gave 13·2 % N (micro-Kjeldahl estimation), which accords with a monoflavianate formula [see Kossel and Gross, 1924]; this value is a little high (calculated 12·1 %) but investigations in this laboratory indicate that a small percentage (about 4 %) of the nitrogen from the flavianate radical yields ammonia in a micro-Kjeldahl estimation.

2·6 g. of arginine monoflavianate were obtained, equivalent to 0·96 g. of arginine and 0·31 g. N, *i.e.* 0·88 % of the total.

No trace of any base other than histidine and arginine could be found in zein.

The remaining solution containing alanine was freed from excess of flavianic acid by continuous extraction with butyl alcohol at 60°; the butyl alcohol extract was extracted with water to ensure complete retention of the alanine in the water-soluble fraction. This was then freed from sulphuric acid and evaporated to a syrup. On granulating with alcohol 12·8 g. of material were obtained, containing 1·68 g. N equivalent to 4·76 % as alanine.

By redissolving this and granulating again with alcohol a sample was prepared which gave 15·18 % N (calculated 15·73 %). A specimen of alanine which was much purer has since been prepared by this method from a subsequent hydrolysis.

B2. *Copper salts soluble in methyl alcohol.*

The methyl alcohol was removed by distillation and the material granulated with acetone; the acetone was removed in a vacuum desiccator and the salts finally dried at 110° for a short time. After confirming that all would redissolve in water and in methyl alcohol, the copper was precipitated by hydrogen sulphide, and the filtrate containing the free acids was neutralised and evaporated to small bulk.

On standing for some days a white crystalline substance separated out, more being obtained on concentrating further.

On recrystallisation from hot water this material was found to be identical with prolylphenylalanine, a peptide first isolated by Osborne and Clapp [1907] from gliadin, and hitherto undiscovered in the hydrolysis products of zein. The specimen prepared contained 10·67 % N (calculated 10·7 %) all in the non-amino-form.

The carbon and hydrogen content of this material agreed with the formula for prolylphenylalanine and derivatives were prepared which confirmed this[1].

2·96 g. of the pure peptide separated at this stage, a further yield being obtained from the valine fraction (see below), giving in all 3·25 g. containing 0·35 g. N, *i.e.* 0·99 % of the total.

The filtrate, from which the peptide had been removed, was evaporated to a syrup and granulated with acetone. The free acids were then extracted with dry absolute alcohol, a large portion remaining insoluble; the soluble portion was repeatedly evaporated to dryness and extracted with alcohol, until all redissolved.

B2. (i) *Free acids soluble in absolute alcohol.*

The solution contained approximately 5 g. N, almost entirely in the non-amino-form. As this fraction was expected to contain the proline, the whole material was dissolved in water and converted into the picrate by Town's method [1928]. Picric acid was added to the boiling solution in sufficient quantity to combine with the non-amino-nitrogen present; on cooling, crystals of proline picrate together with excess picric acid came down; these were filtered off, and on concentrating further more crystals separated. The whole yield was extracted with ether to dissolve excess picric acid (no ether-soluble picrate was found). On recrystallisation from hot water this material gave the characteristic needle-like crystals of proline picrate melting at 148°.

A further yield of proline was obtained from the mother-liquor by precipitation with cadmium chloride. The mother-liquor from the separated picrate would not crystallise any further, so it was decomposed with 1 % sulphuric acid, and extracted with ether to remove the picric acid.

[1] Micro-combustion of this specimen gave a value of 63·8 % C and 7·29 % H (calculated 64 % C, 6·88 % H).
The experimental results are the mean of several estimations.

The aqueous solution was neutralised, evaporated to dryness, and taken up with absolute alcohol; a trace would not redissolve and was added to the main bulk of alcohol-insoluble acids.

A solution of cadmium chloride in 90 % alcohol was added drop by drop to the alcohol extract until no further precipitate formed; this end-point must be carefully watched as the cadmium-proline compound redissolves in excess cadmium chloride. Town found that proline alone is precipitated at this stage.

The precipitate was filtered off and dissolved in water; it was freed from chlorine by precipitation with silver sulphate, the silver and cadmium being removed together by hydrogen sulphide. After neutralising and filtering, the proline in solution was again converted into the picrate, the crystals so obtained being added to the first yield. In all 53·9 g. of proline picrate were prepared, being equivalent to 18·0 g. of proline and 2·19 % N, *i.e.* 6·2 %.

The filtrate, from which the cadmium-proline compound had been separated, was concentrated to remove the alcohol, and taken up with water. The chlorine was removed by silver sulphate and the silver and cadmium by hydrogen sulphide as before. When quantitatively neutral the solution was evaporated to dryness and taken up with absolute alcohol; a small portion did not redissolve and was added to the main bulk of alcohol-insoluble acids.

The alcohol-soluble fraction contained 2·42 g. N (6·85 %); this nitrogen was almost entirely in the non-amino-form.

This fraction was not examined by the author, but was handed to Professor Schryver. In the corresponding fraction from gliadin, Town has succeeded in isolating two piperazine derivatives, *iso*leucine, and some leucine and tyrosine.

B 2. (ii) *Free acids insoluble in absolute alcohol.*

These were redissolved in water, a small portion remaining insoluble; this proved to be prolylphenylalanine, and was added to the main yield of this peptide (see above).

The aqueous solution was reconverted into copper salts to confirm that all would redissolve in methyl alcohol. A small fraction would not redissolve and was filtered off; this precipitate contained 0·02 g. N.

The methyl alcohol was distilled off from the soluble portion, which was then decomposed with hydrogen sulphide, neutralised and evaporated to dryness. The free acids contained 11·6 % N and were obviously a mixture. They were therefore converted into zinc salts, which were dried at 110° and extracted with absolute alcohol [Schryver and Buston, 1926], the extract being evaporated to dryness and taken up with alcohol several times to ensure complete separation.

The greater portion was insoluble, and on decomposition in alkaline solution with hydrogen sulphide, and after neutralising and evaporating, a syrup was obtained which on repeated granulation with alcohol gave 9·15 g. of valine containing 1·01 g. N, *i.e.* 2·86 %.

From this a sample was prepared by repeatedly redissolving and granulating with alcohol, which gave 11·6 % N (calculated 11·96 %).

The zinc salt soluble in absolute alcohol was freed from alcohol by distillation, dissolved in water and decomposed in a similar manner. The free acid on recrystallisation contained 10·51 % N, all in the amino-form, and was found to be identical with hydroxyvaline (10·53 % N)—first isolated from oat protein by Schryver and Buston [1926].

The carbon and hydrogen contents[1] agreed with those of hydroxyvaline and the material gave similar derivatives (*e.g.* benzoyl derivative M.P. 117°, phenyl *iso*cyanate derivative M.P. 145°).

The total yield of hydroxyvaline was 5·04 g. containing 10·51 % N, *i.e.* 0·53 g. N, which is equivalent to 1·5 % in the form of hydroxyvaline; this being the first time it has been isolated from zein.

This completed the separation of the hydrolysis products of zein, which are represented in the following table by their nitrogen contents. The results of the analysis detailed below are in good agreement with those from several previous hydrolyses of zein carried through by the author in the development of the technique.

Table II.

Total nitrogen to be accounted for = 35·33 g.

		Amino-acid	N isolated g.	N as percentage of total N
A.		Aspartic acid	0·68	1·92
		Leucine	5·31	15·05
		Phenylalanine	1·28	3·62
B 1.		Glutamic acid	6·78	19·19
		Tyrosine	0·39	1·14
		Histidine	0·70	1·98
		Arginine	0·31	0·88
		Alanine	1·68	4·76
B 2.	(i)	Prolylphenylalanine	0·35	0·99
		Proline	2·19	6·20
		Alcohol-soluble fraction	2·42	6·85
	(ii)	Valine	1·01	2·86
		Hydroxyvaline	0·53	1·50
C.		Ammonia	7·40	20·95
		Total	31·03	87·89

The total shows 87·89 % of the nitrogen as amino-acids isolated in an approximately pure form; 5·6 % more of the nitrogen was found in precipitates, 93·5 % in all being traced.

Summary.

A simple method is described for the analysis of proteins. The separation is based on the different solubilities of the copper salts of the amino-acids in various solvents.

Experimental details are given of a complete analysis of zein carried through by the process. The results show a marked improvement on those of

[1] Micro-combustion of this specimen gave 44·7 % C and 7·95 % H (average of several determinations). $C_5H_{11}O_3N$ requires 45·1 % C and 8·27 % H.

other methods. In the case of zein 93·5 % of the nitrogen of the hydrolysate was traced.

The author owes much to the late Professor Schryver under whose direction most of the work was done.

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CXXXIV. THE ACTION OF INSULIN ON THE GLYCOGEN DISTRIBUTION IN NORMAL ANIMALS.

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Having shown in a previous communication [Goldblatt, 1929] that insulin can in certain conditions bring about a very large accumulation of glycogen in the liver of a starving animal, it became necessary to inquire whether the additional hepatic glycogen could be accounted for by losses in peripheral carbohydrate. In the former work, in which very young starved rabbits were used, no considerable loss of muscle-glycogen could be demonstrated. In the present work it was determined to examine this matter more closely.

The experimental methods were those already described in the former work. The animals used were very young rabbits and kittens, comparisons being made between litter mates.

Exp. 1. In this experiment the object was to demonstrate in cats the effect which we have observed to occur in young rabbits following the injection of insulin. For this purpose we used kittens from the same litter, which had never had food apart from the mother's milk. The stomach and intestines of such animals after a day or two of starvation are almost empty, and contain no source of carbohydrate. The animals were killed by first stunning and then severing the carotids. Blood was collected straight from the artery into a vessel containing a minimum of oxalate to prevent clotting.

(*a*) Three kittens from the same litter. Starvation 2 days. Never fed apart from mother. Insulin injected subcutaneously.

				Glycogen			
				Liver			
No.	Wt. (g.)	Blood-sugar g. per 100 cc.	Plasma-inorg. P mg. per 100 cc.	(%)	Total (mg.)	Muscle (%)	Remarks
1	510	0·077	7·6	0·71	116	0·15	Control
2	445	"0"	4·4	2·47	378	0·12	3.35 p.m. 1 unit ins. 5.40 p.m. Flaccid and killed
3	460	"0"	6·1	3·39	510	0·18	3.30 p.m. 0·5 unit ins. 6.15 p.m. 0·5 unit 7.40 p.m. Killed. No symptoms

(*b*) Three kittens from the same litter. Starvation 2 days. Never fed apart from mother.

No.	Wt. (g.)	Blood-sugar g. per 100 cc.	Plasma-inorg. P mg. per 100 cc.	Glycogen: Liver (%)	Glycogen: Liver Total (mg.)	Glycogen: Muscle (%)	Remarks
1	319	0·141	—	1·38	123	0·51	Control
2	314	0·041	—	4·13	427	0·45	12.20 p.m. 0·5 unit ins. 4.00 p.m. Convulsion and killed
3	270	"0"	—	2·62	218	0·42	12.20 p.m. 0·2 unit 4.25 p.m. 0·2 unit 5.40 p.m. Killed

Both these experiments confirm our former findings with young rabbits, that insulin produces a very large rise in hepatic glycogen without any simultaneous considerable fall in muscle-glycogen.

(*c*) The following experiment is given in illustration of the fact that the rise in liver-glycogen is not a necessary sequel of a high degree of hypoglycaemia.

Three kittens from the same litter were taken, which, as in the case of the other animals, had never been fed apart from the mother. They were starved for 2 days before the experiment. These animals had developed a severe infection associated with purulent discharge from the eyes. They were obviously severely ill and very probably had a high temperature. They were treated as detailed below.

No.	Wt. (g.)	Blood-sugar g. per 100 cc.	Plasma-inorg. P mg. per 100 cc.	Glycogen: Liver (%)	Glycogen: Liver Total (mg.)	Glycogen: Muscle (%)	Remarks
1	407	0·079	7·2	1·19	176	0·54	Control
2	357	"0"	4·1	0·41	52	0·50	12.20 p.m. 1 unit ins. 2.00 p.m. Flaccid 2.50 p.m. Killed
3	430	"0"	4·1	0·62	99	0·39	12.20 p.m. 1 unit 4.00 p.m. 1 unit 4.50 p.m. Flaccid 5.10 p.m. Killed

It is seen in this case that there occurred a considerable fall in liver-glycogen in the insulinised animals as compared with the control, but no significant change in muscle-glycogen. The effect on blood-sugar and plasma-inorganic P was quite typical. It is difficult to explain the state of affairs here because it is impossible to say whether the infection was equally severe in each animal. It cannot be said that there was an inability to elaborate glycogen in the liver or to retain it, since the control animal had 1·2 %. If it be permissible to compare these animals then it would appear that during the severe infection a condition of things analogous to that in normal rats has been produced. We have shown in a previous communication [Goldblatt, 1929] that insulin does not bring about a rise in liver-glycogen in albino rats and it was suggested that in these animals the mechanism of mobilisation of carbohydrate is adjusted to a very great speed to meet the high peripheral needs of their muscles. In such

animals the effect of insulin seems to be much more marked on the oxidative processes than on the inhibition of glycogenolysis.

Whatever may be the final explanation of this difference in behaviour towards insulin, we feel that no doubt can be entertained that the essential action of the hormone in the intact animal is to exert an antagonism to the release of glycogen from the liver, whilst not preventing the processes of new formation of this substance from endogenous sources.

It might have been expected that the increases of liver-glycogen which we have repeatedly observed following the injection of insulin into the starving animal would be accompanied by marked changes in muscle-glycogen. Indeed, Handowsky [1928] finds that in well-fed rabbits insulin brings about a fall in muscle-glycogen from an average control value of 435 ± 58 to an average of 320 mg. per 100 g. without any fall in lower carbohydrates. Such a fall must evidently be referred to an increased utilisation by the muscles, but in our experiments with starving animals such an effect has not been demonstrable.

In the following three experiments (carried out in collaboration with Dr H. R. Youngman) an attempt was made to see to what extent the loss in peripheral carbohydrate (if any) might possibly account for the increase in hepatic glycogen observed in the insulinised young rabbit.

Exp. 2. Eight young rabbits from the same litter were taken and treated as detailed in the following table.

				Glycogen			
				Liver			
No.	Wt. (g.)	Blood-sugar g. per 100 cc.	Plasma-inorg. P mg. per 100 cc.	(%)	Total (mg.)	Muscle (%)	Remarks
				Control animals.			
1	513	0·122	8·5	1·81	359	0·42	24 hours. Starved
2	515	0·143	7·2	0·54	100	0·39	48 hours. Starved
3	585	0·106	6·4	0·83	166	0·30	72 hours. Starved
4	850	0·171	7·6	10·03	4720	0·71	Not starved
				Insulinised animals.			
5	456	0·020	6·3	4·54	871	0·42	24 hours. Starved 12.10 p.m. 1 unit 2.50 p.m. 1 unit 3.55 p.m. Flaccid and killed
6	485	"0"	5·7	3·20	610	0·23	48 hours. Starved 12.15 p.m. 1 unit 2.50 p.m. 1 unit 5.10 p.m. Killed
7	575	0·060	6·4	2·79	537	0·25	72 hours. Starved 11.40 a.m. 1 unit 2.10 p.m. 1 unit 4.40 p.m. Killed
8	785	0·039	7·1	7·56	2820	0·65	Not starved 11.00 a.m. 2 units 1.30 p.m. 2 units 3.15 p.m. Killed

From these results we make the following calculations. The figures obtained for the control animal are applied *mutatis mutandis* to the insulinised animal starved for the same period, 40 % of the body-weight being allowed for the muscles and one-nineteenth of the body-weight for the blood. We thus obtain an approximation to the probable state of the insulinised animal before treatment with the insulin:

Changes in quantities of glycogen and blood-sugar following insulin.

	Before insulin		After insulin	
No.	Total liver-glycogen (g.)	Total muscle-glycogen (g.)	Total liver-glycogen (g.)	Total muscle-glycogen (g.)
5	0·347	0·766	0·871	0·766
6	0·102	0·757	0·610	0·446
7	0·160	0·690	0·537	0·575
8	3·739	2·229	2·818	2·041

from which we obtain:

No....	5	6	7	8
Change in liver-glycogen...	+ 0·524	+ 0·508	+ 0·377	− 0·921
Change in muscle-glycogen	Nil	− 0·311	− 0·115	− 0·188
Change in blood-sugar ...	− 0·024	− 0·037	− 0·014	− 0·054
Nett change	+ 0·500	+ 0·160	+ 0·248	− 1·163

The nett result of this experiment is, therefore, that in the young rabbit starved for periods of 24, 48 and 72 hours, the increases in liver-glycogen could not be accounted for by the changes occurring in blood-sugar and peripheral glycogen. In the fed animal there apparently occurred a loss of glycogen from all sources, but we cannot insist on this point because the fed animals vary very greatly in their liver-glycogen content even when all precautions are taken. In such animals it seems to us more important to consider that, as in the case of No. 8 above, after almost 6 hours' action of massive insulin doses and with the blood-sugar at 0·039 there still remained 7·56 % of glycogen in the liver, which to all intents and purposes was not available: and further that the presence of large amounts of assimilable foodstuffs in the gut was unable to prevent the appearance of hypoglycaemia.

If we take the view that muscle-glycogen is in an irreversible condition and so cannot contribute to circulating carbohydrate, then the calculation given above becomes superfluous, since it could not contribute to liver-glycogen either.

Exp. 3. This experiment was exactly similar to the last one. Four young rabbits from the same litter were taken and treated as below.

				Glycogen			
				Liver			
No.	Wt. (g.)	Blood-sugar mg. per 100 cc.	Plasma-inorg. P mg. per 100 cc.	(%)	Total (mg.)	Muscle (%)	Remarks
				Control animals.			
1	490	0·177	8·8	5·49	1450	0·86	Unstarved
2	557	0·113	6·3	0·37	60	0·49	48 hours. Starved

No.	Wt. (g.)	Blood-sugar mg. per 100 cc.	Plasma-inorg. P mg. per 100 cc.	Glycogen: Liver (%)	Glycogen: Liver Total (mg.)	Glycogen: Muscle (%)	Remarks
				Insulinised animals.			
3	450	0·027	6·0	6·54	1534	0·67	Unstarved 10.35 a.m. 1 unit 1.00 p.m. 1 unit 2.40 p.m. Flaccid and killed
4	532	"0"	4·8	2·17	463	0·35	48 hours. Starved 11.00 a.m. 1 unit 1.25 p.m. 1 unit 2.00 p.m. Flaccid and killed

Applying a similar calculation to that in the last experiment we get:

No.	Before insulin: Total glycogen in liver (g.)	Before insulin: Total muscle-glycogen (g.)	After insulin: Total glycogen in liver (g.)	After insulin: Total muscle-glycogen (g.)
3	1·300	1·548	1·534	1·426
4	0·079	1·043	0·463	0·745

from which we obtain:

No.	3	4
Change in liver-glycogen...	+ 0·234	+ 0·384
Change in muscle-glycogen	− 0·122	− 0·298
Change in blood-sugar ...	− 0·040	− 0·032
Nett change	+ 0·072	+ 0·050

When it is considered that under the influence of insulin there occurs an increase in peripheral oxidation of carbohydrate, then it becomes still less possible to account for the increase in liver-glycogen by peripheral loss of glycogen.

In the next experiment the analysis of the animals included the estimation of free sugar of the tissues by alcohol extraction as used in previous work [Goldblatt, 1929].

Exp. 4. Six young rabbits from the same litter were taken and treated as below.

	Controls 1	Controls 2	Controls 3	Insulinised 4	Insulinised 5	Insulinised 6
Wt. (g).	553	567	655	525	550	653
Blood-sugar mg. per 100 cc.	0·162	0·141	0·137	0·035	0·020	"0"
Plasma-inorg. P mg. per 100 cc.	8·7	8·2	8·9	9·1	8·7	7·4
Glycogen:						
Liver %	4·94	0·91	0·33	4·24	3·60	2·27
Liver total g.	1·240	0·217	0·067	1·100	1·093	0·500
Muscle %	0·62	0·67	0·38	0·37	0·45	0·36
Free sugar as glucose:						
Liver %	0·76	0·73	0·61	0·66	0·67	0·51
Muscle %	0·37	0·30	0·31	0·28	0·25	0·38

Treatment of the above animals.

No. 1. Unstarved.
2. Starved 24 hours.
3. Starved 48 hours.
4. Unstarved and insulinised as below:
12.25 p.m. 1 unit.
4.00 p.m. 1 unit.
4.50 p.m. Flaccid and killed.
5. Starved 24 hours.
12.30 p.m. 1 unit.
3.55 p.m. 1 unit.
5.35 p.m. Convulsion and killed.
6. Starved 48 hours.
12.28 p.m. 1 unit.
2.30 p.m. Convulsion.
2.55 p.m. Convulsion and killed.

Constructing a table as before by applying control values to the insulinised animals, we obtain the following:

	Glycogen		"Free sugar"	
No.	Total in liver (g.)	Total in muscles (g.)	Total in liver (g.)	Total in muscles (g.)
	Before insulin.			
4	1·283	1·302	0·197	0·777
5	0·276	1·474	0·221	0·660
6	0·072	0·992	0·134	0·812
	After insulin.			
4	1·100	0·777	0·171	0·588
5	1·093	0·990	0·203	0·550
6	0·500	0·950	0·112	0·992

from which we obtain:

No.	4	5	6
Change in liver-glycogen	− 0·183	+ 0·817	+ 0·428
Change in muscle-glycogen	− 0·525	− 0·484	− 0·042
Change in free sugar of liver (as glycogen)	− 0·223	− 0·015	− 0·019
Change in free sugar of muscle (as glycogen)	− 0·162	− 0·095	− 0·167
Loss in blood-sugar	− 0·035	− 0·040	− 0·047
Nett change	− 1·138	+ 0·183	+ 0·153

These figures, based on comparisons of animals from the same litter and under controlled conditions, show an excess formation of glycogen in the liver of starving insulinised animals not explicable by differences in muscle-glycogen, free sugar of the tissues or blood-sugar. If, however, we had based our calculations on average values obtained from animals of various litters, no approach to a balance would have been obtained. The following table illustrates this by giving the glycogen content of liver and muscle in young rabbits starved for 24 hours and treated with insulin for varying times and with different doses. The majority of the insulinised animals were killed at the onset of convulsions.

Glycogen content of young rabbits starved for 24 hours.

Controls			Insulinised animals		
Wt. (g.)	Liver (%)	Muscle (%)	Wt. (g.)	Liver (%)	Muscle (%)
667	0·55	—	340	0·79	0·17
493	0·82	—	415	1·63	0·25
546	0·81	0·28	414	1·76	0·18
563	0·62	0·29	523	1·40	0·08
338	0·33	0·12	473	0·95	0·23
397	0·32	0·16	479	0·92	0·20
373	0·31	0·21	427	2·03	0·15
565	0·60	0·06	366	1·56	0·36
427	0·15	0·19	535	1·28	0·28
384	0·27	0·43	538	0·78	0·32
328	0·34	—	535	1·19	0·32
451	0·59	0·30	492	3·62	0·30
567	0·91	0·67	587	4·43	0·23
505	0·32	0·50	550	3·60	0·45
			556	4·54	0·42
Averages	0·44	0·27	416	1·49	0·21
			Averages	2·00	0·26

It is clear that such a series gives no grounds for considering that peripheral glycogen may be a source of the increase in liver-glycogen. But even when, as in the above calculations, any changes in muscle-glycogen in individual litters are deducted from the changes in liver-glycogen, there still remains an excess unaccounted for.

It is also to be noted that in the unstarved animals insulin seemed to produce in the majority of cases a loss of carbohydrate from all sources. Control is impossible with animals taken straight from feeding, but, although the figures are only suggestive, taken in conjunction with our former findings with animals given a known amount of glucose *per os* [Goldblatt, 1929], it does appear that in the actively absorbing animal insulin may produce a general loss of carbohydrate in all situations. But in the starved animal there occurs an (as yet) unaccountable new formation of glycogen.

Frank *et al.* [1927] using rabbits starved for periods varying from 36 hours to 4 days, found (average of 7) a liver-glycogen of 0·165 % and a muscle-glycogen of 0·127 %: after a dose of 0·1 unit of insulin they found in similar animals (average of 6) 1·53 % of glycogen in the liver and 0·353 % in the muscles. These authors conclude that the rise in liver-glycogen must have originated in body protein. There is little evidence of an increased protein catabolism due to insulin.

Kerr and Krikorian [1929], using dogs starved for 24 and 48 hours, could find no increase in the non-protein nitrogenous constituents of the blood, even when large falls in blood-sugar were produced by insulin.

Sokhey and Allan (1924), using dogs starved for 8 to 20 days, found an increase of about 20 % in total N excretion 6 hours after the injection of insulin, this increase not being associated with an increased output of creatinine. When, however, the animals were well fed or given glucose in addition to the insulin, the nitrogen excretion diminished. In these experiments the

constancy of creatinine excretion indicates a constancy of tissue breakdown, a finding which makes it difficult to see what the origin of the increased nitrogen could have been. Attempts to demonstrate an increased transference of non-protein nitrogen in the blood of insulinised animals have, in our hands, been unsuccessful (*vide infra*).

The possibility that fat might be the source of the newly formed glycogen in our animals must not be lost sight of, but we have no direct evidence of this.

As the matter stands at present it can only be said that our evidence indicates an endogenous and non-carbohydrate source for the glycogen which has been observed to accumulate in the livers of insulinised starving young rabbits. This accumulation, in some peculiar way, is not immediately available to the animal even when it is in extreme need of circulating carbohydrate as at the onset of hypoglycaemic convulsions.

The following experiments were carried out to determine whether an increase in liver-glycogen could be obtained in insulinised young rabbits starved for several days. In our former experiments no special precautions were taken to ensure that the stomach and intestines should be quite empty at the time of experiment. Thus, the animal ingested a certain amount of its faeces before this material fell through the mesh of the cage, some of this being found in the stomach after death. But as this applied to both the controls and the insulinised animals, the validity of our findings remains unaffected. However, in the following two experiments special precautions were taken to prevent the ingestion of faeces but allowing water *ad lib.* In every case the stomach and small intestine were empty of all solid material, there being usually a quantity of fluid in the stomach giving a strong reaction for hydrochloric acid.

Exp. 5. Four young rabbits from the same litter were starved for 5 days, fluid *ad lib.*, with special precautions to prevent ingestion of faeces. Treatment as below.

		Glycogen			
No.	Wt. (g.)	Liver (%)	Total liver (mg.)	Muscle (%)	Remarks
1	652	1·972	375	0·295	Control
2	552	1·621	226	0·295	Control
3	572	2·863	506	0·533	12 noon. 2 units 2 p.m. 2 units 2.30 p.m. Convulsion and recovery 3.00 p.m. 2 units 3.15 p.m. Convulsion and killed
4	567	2·758	524	0·447	12 noon. 2 units 2.00 p.m. 2 units 3.00 p.m. 2 units 4.00 p.m. 2 units 4.10 p.m. Convulsion and killed

It thus was quite clear that the same phenomenon of glycogen synthesis in the liver as a result of insulin action occurred with the longer starvation and the additional precautions. In addition, the muscle-glycogen showed a surprising increase in the treated animals. Such increases in muscle-glycogen

have, in our experience, been very exceptional, but the results of Frank *et al.* [1927] seemed to show that a stimulus to glycogen formation could be obtained even in muscle. But as their muscle-glycogen values in the insulinised animals varied from 0·03 % to 0·93 % not much weight can be attached to them. In our series of young animals, however, the correspondence is much closer and, in view of the very high doses of insulin used, the high muscle values seem to us significant. It may be that the longer period of starvation was the factor responsible for this different reaction to insulin.

In the next experiment a more extensive analysis of the blood of the insulinised animals was carried out to see if any transference of a significant substance could be detected during the deposition of liver-glycogen resulting from the action of insulin. The methods of Folin were used for plasma-creatinine and amino-acid-N. No proportionality of developed colours was assumed beyond a range of 2 mm. on the Duboscq colorimeter, so that a large number of standards had to be prepared. Cholesterol was estimated as an index of circulating lipoid material by the method of Myers and Wardell. Non-protein-N in the plasma was determined by precipitation with trichloroacetic acid and the micro-Kjeldahl method, blanks being run on the reagents.

Exp. 6. Seven young rabbits, litter mates, were starved for 3 days, taking precautions that no faecal matter was ingested. Treatment as detailed below.

No.	Wt. (g.)	Blood-sugar g. per 100 cc.	Plasma-inorg. P mg. per 100 cc.	Plasma-creatinine mg. per 100 cc.	Plasma-amino-acid N per 100 cc.	Whole blood-cholesterol mg. per 100 cc.	Plasma-non-protein N mg. per 100 cc.
1	583	0·131	6·2	2·1	3·6	114	—
2	601	0·113	5·9	2·2	—	107	48
3	549	0·087	5·5	—	3·4	92	47
4	585	0·083	5·5	2·4	—	105	45
5	646	0·102	5·6	1·9	—	126	42
6	593	0·045	4·2	2·1	2·6	106	34
7	630	0·039	4·9	1·8	—	104	30

Analyses for glycogen in the above animals.

No.	Liver (%)	Total in liver (mg.)	Total in liver per 100 g. animal (mg.)	Muscle (%)	Remarks
1	1·280	240	40	0·403	Control
2	1·204	232	39	0·329	Control
3	1·136	165	30	0·474	10.25 a.m. 0·2 unit 1.30 p.m. Killed
4	1·857	335	57	0·353	10.25 a.m. 0·5 unit 2.00 p.m. Killed
5	1·281	328	51	0·351	10.26 a.m. 1·0 unit 2.30 p.m. Killed
6	1·380	242	41	0·617	11.25 a.m. 1·5 units 3.10 p.m. 2·0 units 4.35 p.m. Flaccid and killed
7	2·390	483	77	0·520	11.25 a.m. 2·0 units 3.10 p.m. 2·0 units 4.10 p.m. Convulsion and killed

Analysis of the findings in this experiment shows that no explanation of the glycogen synthesis is to be found in the blood constituents. Estimation of the protein fractions of the blood have also given us no indication for the explanation of the source of the new glycogen deposited in the liver. It will be seen that in every case except No. 3 and No. 6 the glycogen percentage in the liver was higher than in the controls and we have no doubt, from the large number of our former experiments, that, had the action of the insulin been allowed to continue, a greater synthesis of glycogen would have occurred. The muscle figures showed no loss of peripheral glycogen in comparison with the controls; and in Nos. 6 and 7, as in Exp. 5, there was a suggestion that possibly an increased synthesis of glycogen was occurring in the muscles also.

Since so considerable a quantity of glycogen was present in the liver and tissues of the untreated animals, it is clear that the process of endogenous glycogen formation goes on at a rate considerably greater than that sufficient to supply energy necessities. There appears to exist a kind of metabolic hysteresis, the oxidative mechanisms lagging somewhat behind glycogen synthesis. Regarded from this point of view, glycogen would seem to be a true storage material. Further, the continued deposition of this substance even during periods of dire need of calorigenic material appears good evidence that it is a necessary intermediate stage for the utilisation of tissue metabolites.

If this is in fact the case then, in conditions where little or no glycogen is formed, as in pancreatic diabetes, we are reduced to considering a direct utilisation of a fatty acid as the source of energy. The fact that in diabetes the immediate effect of insulin is to bring about a formation of glycogen, both in liver and muscles, from the excessive quantities of sugar present, is, it seems to us, strongly in favour of glycogen being a necessary intermediary in carbohydrate metabolism. As has already been pointed out, the processes which in the normal starving animal lead to the deposition of glycogen are in advance of those governing oxidation of carbohydrate. In this way a certain excess of the colloid is assured in the liver. The deposition of this excess is associated with the blood picture which is called normal in the fasting animal. Differences in the degree of this excess depend on the efficiency of control of those endocrine organs which have to do specially with carbohydrate metabolism.

Now, whereas in the diabetic animal the processes of gluconeogenesis are accompanied by profound blood changes (hyperglycaemia, lipaemia, acidosis, ketosis), these occur in the starving normal animal only to a very slight extent (excluding hyperglycaemia). In the normal rabbit we have not found any of these changes even after 9 days' starvation. It is almost certain that this difference is due to the inability of the diabetic to deposit glycogen.

Administration of insulin, whilst bringing the blood constituents of the diabetic back to normal, does not lower the values in a normal animal, except in the cases of sugar and inorganic P. In spite of this an increased synthesis of glycogen is brought about in both, and in the case of the normal rabbit we have been unable to account for it by the changes in the carbohydrate already

existing in the body. It is therefore considered that insulin can produce an actual stimulus to the processes of glycogen formation in normal animals in addition to opposing the mechanism of glycogenolysis in the liver.

Where, as in the diabetic, large quantities of tissue and blood-sugar are available, the formation of liver-glycogen resulting from the administration of insulin would probably be easily explicable, but it might not be so readily explicable if the administration of insulin were continued after the excess of carbohydrate had been disposed of for energy purposes, at which time the previously diabetic animal would have approached closely to the starving normal.

The theory that insulin inhibits gluconeogenesis (by which we understand the production of carbohydrate from non-carbohydrate sources) is only admissible in so far as the injection of the hormone promotes the formation of glycogen from the excessive amounts of sugar already present, since as is generally recognised the presence of adequate concentrations of glycogen in the liver inhibits tissue catabolism. The fact that the greatest degrees of new formation of glycogen which we have observed to follow injection of insulin occurred in cases showing the greatest degrees of hypoglycaemia suggests to us that this phenomenon is the result of an attempt to deal with the extreme crisis which the organism is undergoing. This type of reaction is already known to occur in the diabetic animal, which breaks down its tissues in a futile attempt to make good its losses of carbohydrate. If this process can take place in the diabetic organism which seems quite unconscious of the large amounts of sugar bathing its cells, it seems to us not improbable that the normal cell may react similarly to so real a deficiency of circulating sugar as occurs in hypoglycaemia, by increasing its rate of formation of glycogen.

SUMMARY.

1. The increases in liver-glycogen observed to follow the injection of insulin into starving young rabbits cannot be explained by changes occurring in glycogen and "free sugar" in other situations. This is considered the more important since the majority of experimental work tends to show that muscle-glycogen is irreversible and so cannot contribute to circulating carbohydrate.

2. These accumulations of liver-glycogen are not associated with changes in blood constituents other than diminution in blood-sugar and blood-inorganic P.

3. It is considered that under the described conditions glycogen formation from endogenous sources is definitely increased.

It is a pleasure to express my gratitude to Prof. H. MacLean for his advice and encouragement.

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CXXXV. STUDIES IN PHOTOSYNTHESIS.

I. THE FORMALDEHYDE HYPOTHESIS.

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Introduction.

Since 1870, when Baeyer put forward the suggestion that formaldehyde was the first stage in the photosynthetic process in the green leaf and that this substance was subsequently polymerised to reducing sugars, numerous investigators have from time to time attempted to test the validity of this hypothesis and in the main have pronounced in its favour on no very convincing evidence.

The experimental work on this aspect of plant physiology has been conducted along several different lines, such as feeding plants with formaldehyde or its derivatives, or testing leaves for formaldehyde, and also by means of *in vitro* experiments.

According to Loew [1889] and Bokorny [1891] the alga *Spirogyra*, when fed with the sodium bisulphite compound of formaldehyde, forms starch, while Boitreux [1920] and Moore and Webster [1920] have put forward the claim that *Trichoderma viridis* can assimilate free formaldehyde. A similar claim was made for the higher green plants some years previously by Baker [1913] and also by Grafe and Vieser [1909, 1911] provided that the concentration of the aldehyde was not too high and that there was sufficient illumination. On the other hand, formaldehyde vapour readily oxidises in the air to give formic acid, so that if these results prove anything at all they show that formic acid is the first product of photosynthesis.

The utilisation of a compound by a leaf is no proof that it forms an intermediate stage in its metabolic activities, and a large number of substances are now known, such as glycerol, which can be used by the green leaf for the preparation of starch.

The difficulty of obtaining convincing proof of the truth of the formaldehyde hypothesis has led to the adoption of *in vitro* experiments. Baly [1921, 1927, 1929], who is at present the chief investigator in this particular field of photosynthesis, claims that the processes *in vitro* and *in vivo* are similar. He states that when solutions of carbon dioxide are exposed to ultra-violet radiation

appreciable amounts of formaldehyde are produced, and further asserts that if a coloured catalyst, such as cobalt or nickel carbonate, be present, this process takes place in ordinary light with the production of reducing sugars. This latter change, *i.e.* when coloured catalysts are present, is said to proceed at a rate directly proportional to rise of temperature for the lower range of temperature values (5–31°), but this proportionality ceases above 31°, and there is a rapid decrease in yield. For a complete review of this work see Barton-Wright [1930].

The chief disability under which the formaldehyde hypothesis labours is that this compound is highly toxic to the living cell, and if it were produced in any quantity would quickly cause death. This difficulty has been answered by the assumption that the formaldehyde is polymerised as fast as it is produced, while Baly considers that the ordinary form of the aldehyde, $H-C\begin{matrix}O\\H\end{matrix}$, is not produced in the process, but that the tautomeric, or, as he prefers to call it, "active" aldehyde, H—C—OH, is formed, and it is on this account that it cannot be detected by the usual tests.

The most convincing evidence for the validity of the formaldehyde hypothesis appeared in a recent paper by Klein and Werner [1926]. These investigators employed the reagent dimedon (dimethylhydroresorcinol) which has been so successfully used by Neuberg and his co-workers in their work on the elucidation of the chemical mechanism of alcoholic fermentation.

Dimedon forms a delicate test for aldehydes and readily reacts with these bodies to give insoluble condensation products.

With formaldehyde and acetaldehyde the reaction is particularly delicate, and we have been able to detect as small amounts of formaldehyde as 1 part in 100,000 of water. Moreover, it is a simple matter to distinguish between formaldomedon and acetaldomedon, since these condensation products differ in crystal form and melting point.

Klein and Werner employed a number of different plants in their experimental work, aquatics such as *Elodea canadensis*, *Myriophyllum* (sp.), *Potamogeton* and others, and also ordinary mesophytes and succulents.

In the case of aquatics, the plants were placed in flat dishes containing nutrient solution together with 10–20 % of calcium bicarbonate to supply carbon dioxide, and dimedon in concentration of 1 in 1000 was then added. This proportion of dimedon was said to reduce only slightly the assimilation rate. With mesophytes, cut shoots were placed in the shorter arm of an unequal U-tube and a solution of dimedon (1 in 2000) was forced through the stem by means of a mercury column. It was discovered that the dimedon had a narcotic effect on the leaves and apparently caused the stomata to close. The latter were kept open artificially by means of a 0·1 % solution of sodium chloride. In succulents, such as *Sempervivum*, the dimedon solution was directly injected into the assimilating tissues by means of a Pravz syringe. Here again the stomata tended to close and were kept open by the addition of weak sodium chloride solution. It was found that stronger solutions of

dimedon (1 in 200 and 1 in 500) could be used with succulents without materially reducing the rate of assimilation.

The most extensive experiments, however, were conducted with aquatics, especially *Elodea canadensis*. The plants were placed in flat dishes in the manner described above, together with nutrient medium containing calcium bicarbonate and dimedon, and allowed to assimilate for a period of 6–8 hours under a constant light intensity of 40,000–100,000 lux at a temperature of 20°. The condensation product of formaldehyde and dimedon (formaldomedon) was then detected in the external solution and little was found in the plants themselves.

It was ascertained that formaldomedon was only produced in the light and in the presence of carbon dioxide. In the dark, acetaldomedon was formed instead. Again, narcotics, such as potassium cyanide and phenylurethane, in dilute concentration, prevented the production of formaldomedon, and only a very small amount of acetaldomedon was produced.

This work appeared to lend definite support to a long-standing hypothesis. It therefore appeared necessary to us to confirm it in every particular, more especially as we could not understand why the formaldomedon was principally present in the external medium, so far as aquatics were concerned, and only a trace was to be found in the assimilating cells. A further point that made repetition necessary was the fact that nowhere throughout the course of their investigation did Klein and Werner make any attempt to control the p_H of their medium—an important point in an investigation of this nature. We have accordingly repeated this work and the results are recorded below.

Experimental.

The plant used in this investigation was *Elodea canadensis*, which was collected from a pond at Greenford, Middlesex, and kept in large tanks in Knop's medium diluted to 1 in 20, together with some mud from the bottom of the pond. The tanks were kept continuously aerated by a special device. With this arrangement the plants remained in a perfectly healthy condition and grew vigorously.

Preparation of dimedon. The dimedon was prepared by Vorlander's [1893] method with only slight modification.

Narcotic effect of dimedon on assimilation. Klein and Werner have stated that dimedon has a narcotic action on the rate of assimilation, but they give no exact figures for this depressant effect. The matter was quantitatively investigated here by means of a Wilmott's "bubbler." Cut shoots were placed in Knop's solution (previously diluted to 1 in 20), containing 0·6 % of sodium bicarbonate and exposed to a light intensity of 1400 candle-power at a distance of 2 feet. Excess of heat rays was removed by a water screen and the temperature was maintained at 19° ± 1. In each experiment the shoot was allowed to assimilate normally for 2 hours and then the necessary amount of dimedon was added and the fall in the rate of assimilation observed. A representative experimental result is given in Table I.

Table I.

Time	Bubbles per min.	
11.00 a.m.	—	Culture solution, 0·6 % $NaHCO_3$ alone
11.30 a.m.	41	
12.00	41	
12.30 p.m.	40	
1.00 p.m.	41	Dimedon added (0·1 %)
1.30 p.m.	31	
2.00 p.m.	30	
2.30 p.m.	29	
3.00 p.m.	33	
3.30 p.m.	34	

p_H of solution 7·4

In every case that was tried it was discovered that the addition of dimedon produced a markedly depressant effect on the rate of assimilation.

Klein and Werner state that dimedon reduced the p_H of their nutrient medium (the nature of which they do not state) from 8·0 to 4·5. We found that a 0·1 % solution of dimedon reduced the p_H of Knop's solution containing 0·6 % of sodium bicarbonate from 8·6 to 7·4. In all subsequent work, therefore, the p_H of our various culture media was maintained at this latter value by suitable buffering.

For the actual experimental work dealing with the alleged production of formaldehyde, Klein and Werner's methods were closely followed. A number of shoots of *Elodea*, approximately 10 cm. long, were cut and placed in flat dishes with the Knop's mixture containing bicarbonate and dimedon, and exposed to a constant light intensity of 1400 candle-power for 6–8 hours at a distance of 18 in. Excess of heat rays was removed by suspending between the source of light and the plants a water screen through which a continuous stream of water was maintained. With this arrangement the temperature remained at $20° \pm 1$ for the whole experimental period. The p_H of the medium was tested every hour and was brought back to 7·4 by the addition of a suitable amount of buffer (p_H 2·6). A control experiment was run in the dark, the p_H again being kept at 7·4. We deviated, however, from Klein and Werner at this stage, in that we also exposed to light under the same conditions a nutrient solution containing bicarbonate and dimedon, but no plant, and a similar solution was placed in the dark.

At the end of the assimilation period, the procedure described by Klein and Werner was followed. A little sodium carbonate was added to the nutrient solutions to insure that all the formaldomedon had passed into solution, as this substance is very insoluble in acid or neutral media, but is readily soluble in alkaline solutions. The solution was then filtered and acidified with hydrochloric acid and extracted with light petroleum (100 cc. to 1 litre of solution) for several hours on a mechanical shaker. The solvent was separated and twice washed with a saturated solution of sodium chloride and evaporated to dryness under reduced pressure at 40°. A mass of white crystals was obtained. This consisted of a mixture of dimedon and formaldomedon in the case of plants

exposed to light and, according to Klein and Werner, unchanged dimedon and acetaldomedon for plants that had been allowed to remain in the dark.

To separate the condensation product from unchanged dimedon, Klein and Werner dissolved the crystalline residue in 3 cc. of a 10 % solution of potassium carbonate and then slightly acidified with phosphoric acid. This mixture was placed in a small retort and distilled over a sand-bath at 200° into a saturated solution of dimedon. According to Neuberg, acetaldomedon is hydrolysed by boiling water, whereas formaldomedon is resistant and only hydrolysed by acid. Klein and Werner claimed that by this method formaldomedon distilled over from the mixture and was reprecipitated as formaldomedon in the container.

We repeated this procedure a number of times with uniformly unsuccessful results. In no case did any formaldehyde pass over and become precipitated in the container. In order to see whether any hydrolysis did in fact take place or whether our experimental conditions were possibly not correct, pure formaldomedon was prepared and treated in the same way. Here again, no formaldehyde passed over and the product appeared to be quite resistant to hydrolysis by phosphoric acid. Nor were we able to confirm Neuberg's statement that acetaldomedon is hydrolysed by boiling water. It may be mentioned in passing, that with the distillation method employed by Klein and Werner, unless very great care is exercised, sucking-back takes place and the retort explodes.

We therefore modified Klein and Werner's method of separation. It was found by treating mixtures of formaldomedon and dimedon with boiling water that these two products could be quantitatively separated. The dimedon passed into solution and could be filtered off and the formaldomedon was left as an insoluble residue. Applying this method the following results were obtained.

(1) Plants exposed to light in a nutrient solution together with sodium bicarbonate and dimedon; an examination of the external solution revealed the presence of a small amount of formaldomedon, M.P. 180–185°.

(2) Plants left in the dark with dimedon and sodium bicarbonate; no formaldomedon or acetaldomedon produced, dimedon entirely recovered.

(3) Nutrient solution plus sodium bicarbonate and dimedon exposed to the light, but no plant present; a small amount of formaldomedon produced.

(4) As (3), but the solution placed in the dark; dimedon entirely recovered, no formaldomedon formed.

The effect of anaesthetics was also investigated. Plants were allowed to assimilate in the presence of phenylurethane and potassium cyanide. In solutions of these narcotics of strength 0·1 to 0·0005 % no visible assimilation was found to occur, but our solutions, contrary to the statements made by Klein and Werner, still yielded formaldomedon at the end of the experimental time.

An attempt was also made to see whether any formaldomedon was deposited within the tissues of the *Elodea*. Cut shoots were allowed to assimilate

in the presence of dimedon for 6–8 hours. The tissues were now rubbed up with 1 g. of lead acetate and 3 g. of barium hydroxide, according to the details given by Klein and Werner, so that the mixture was strongly alkaline. 100 cc. of water were added and the whole allowed to stand for 24 hours. At the end of this time, the solution was filtered, acidified with hydrochloric acid, shaken with the precipitate that had formed and again filtered. The solution was finally extracted with light petroleum in the manner described above and evaporated under reduced pressure. Although this experiment was repeated some ten or fifteen times, in no case could we find a trace of either dimedon or formaldomedon in the extract.

Discussion.

It will be seen from these results that the production of formaldomedon in the external solution surrounding the plant is no criterion that formaldehyde is the first product of photosynthesis in the living plant. Bicarbonate solutions in the presence of light produce appreciable amounts of the aldehyde and this process is independent of the assimilation mechanism of the plant. Our results therefore indicate that in aqueous solutions of either bicarbonate or carbon dioxide the following equilibrium exists:

$$CO_2 + H_2O \rightleftharpoons H . CH : O + O_2.$$

In the presence of such a substance as dimedon, which removes formaldehyde from the system as an insoluble precipitate as soon as it is formed, the equilibrium is pushed towards the right half of the equation. Furthermore, the fact that, unlike Klein and Werner, we were still able to detect the presence of formaldomedon in the external solution in the presence of anaesthetics such as phenylurethane, which suppresses all visible assimilation, shows very clearly that the production of formaldehyde in these circumstances has no connection with the photosynthetic process in the living plant.

The experiments carried out by Klein and Werner on land plants, in which the dimedon was conducted to the assimilating cells *via* the transpiration stream, have not been repeated by ourselves. These workers have put forward the claim that in all cases they were able to isolate formaldomedon from leaves exposed to light. This experimental work, however, is open to the strong objection that dimedon undoubtedly exerts a marked toxic action on the plant, and Klein and Werner themselves admit that its presence caused the stomata to close. It may well be on this account that, if any formaldehyde were produced, it would be a direct product of decomposition processes taking place in the living cell due to the toxic action of the dimedon. It is a well-known fact that the decomposition of the chlorophyll pigments can, in certain circumstances, give rise to formaldehyde; a result which has led some investigators to make the claim that this is proof that formaldehyde is a product of assimilation, whereas in reality they have been dealing with a decomposition by-product. Lastly, it will be remembered that we were unable to find any trace of formaldomedon in the leaves of *Elodea*.

Although the formaldehyde hypothesis has the merit of simplicity, which has probably been the prime cause of its wide popularity, no work has as yet convincingly shown that formaldehyde is produced normally in the green leaf or that it plays any part in the photosynthetic process of the living plant.

Summary.

The claim made by Klein and Werner to have shown by the use of dimedon that formaldehyde is the first product of photosynthesis has been re-investigated. It is shown that there is no experimental foundation for this statement, and that the production of formaldehyde in their solutions is due to the action of light on carbon dioxide and bicarbonates and is independent of the photosynthetic mechanism of the living plant.

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CXXXVI. STUDIES IN PHOTOSYNTHESIS.

II. THE FIRST SUGAR OF CARBON ASSIMILATION AND THE NATURE OF THE CARBOHYDRATES IN THE NARCISSUS LEAF.

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Introduction.

In spite of the large number of investigations that have been made from time to time on the nature of the carbohydrates in the green leaf and the sequence of events that leads to their synthesis, there is at the present time no unanimity of opinion with regard to the nature of the first sugar formed in photosynthesis. Such an investigation is beset with a number of difficulties. The chemistry of the mesophyll cells is complex, for within them protein synthesis and respiration are taking place at the same time and the matter is still further complicated by the translocation of these substances to the storage organs.

The products of photosynthesis, either direct or indirect, are hexoses, disaccharides and polysaccharides. It would be expected on purely chemical grounds that the simplest members of this series, the hexoses, would be the first sugars formed in the assimilating organs of the green plant and that these in turn would suffer condensation to disaccharides. Nevertheless, the majority of workers on this problem of plant physiology have reported in favour of the hypothesis of sucrose being the first sugar of photosynthesis. Sucrose is a product peculiar to the plant world and has a wide distribution in every group of green plants.

It is not proposed here to give a detailed review of previous work on this subject, a large amount of which is of little value for a variety of reasons. The first adequate investigations on the quantitative relationships of the sugars of the leaf were carried out by Brown and Morris [1893] on *Tropaeolum majus*. In such work as this, it is very necessary that the leaf material should be killed instantaneously so that enzymic changes are prevented. Brown and Morris killed their material by drying in a steam oven between 75° and 80°. The carbohydrate values were calculated as percentages of the dry weight and the carbohydrates found were starch, maltose, sucrose, glucose and fructose.

From the variations in the amount of sucrose in leaves and the small

amounts of glucose and fructose found under different conditions Brown and Morris claimed that sucrose and not hexose was the first sugar of photosynthesis. It was considered that when the concentration of sucrose reached a certain value it was converted into starch, while for purposes of translocation it was first inverted to glucose and fructose and removed as such to the roots.

Stiles and Jørgensen [1916] have pointed out in a critical discussion of this work that the values recorded by Brown and Morris for the glucose and fructose fractions of their preparations are extremely doubtful. Davis, Daish and Sawyer [1916] were quite unable to find maltose in their preparations from *Tropaeolum* leaves and concluded that the method of killing by heating in a steam oven had resulted in a certain amount of degradation of the starch by diastatic enzymes.

Parkin [1911], from his investigations of the monocotyledonous plant, *Galanthus nivalis* (snowdrop), also supported the views of Brown and Morris that sucrose and not hexose was the first sugar of photosynthesis. In monocotyledonous leaves, the complications introduced by the presence of starch are eliminated, for, as a general rule, the leaves of this group of plants do not form starch and only a quite negligible amount is to be found in the guard-cells of the stomata.

Parkin found that as a general rule the percentage of hexoses remained fairly constant in amount during any single day in the spring, whereas the sucrose exhibited marked fluctuations, increasing during the day and diminishing during the night. With advance in the season the proportion of hexose to sucrose was found to increase. Parkin concluded that his values justified the suggestion of Brown and Morris that sucrose was the first sugar of carbon assimilation. Although this view is possibly justified from the experimental figures, the intervals of time that were allowed to elapse before each observation was taken were far too long to allow of deductions of this nature being made from them.

A highly elaborate attempt to settle this problem was carried out at the Rothamsted Experimental Station by Davis, Daish and Sawyer [1916] on the leaf of the mangold. These workers estimated sucrose, maltose, glucose, fructose, starch, pentoses and pentosans. The leaf material, after the midribs had been removed, was killed by throwing it into boiling 95 % alcohol containing a little ammonia.

Collections of the leaves were made at 2-hourly intervals over 24-hour periods. Three such determinations were made, in August, September and October respectively. The mangold leaf was found to contain starch only in the early stages of growth. In each of these experimental runs the sugars of the leaf lamina and petiole were treated separately and the results were expressed as a percentage of the dry weight.

In general terms it was found that in the early summer the soluble sugars (hexoses and sucrose) increased in the light to a maximum about noon and then fell off fairly regularly till the following dawn, and the concentration of sucrose

was always in excess of the hexose values and the percentage of pentosans remained fairly constant. No maltose was found to be present.

In the second experimental run (September) the hexoses were found to be in excess of sucrose and both the curves showed maxima at 2 p.m., 6 p.m. and 2 a.m. The results obtained in October were much the same as those obtained in September. Like Brown and Morris and Parkin, Davis, Daish and Sawyer were of the opinion that sucrose was the first formed sugar and that inversion of the sucrose to glucose and fructose took place prior to translocation, since hexoses were found to be in excess of sucrose in the petiole.

The problem of the first sugar has been investigated from a different aspect by Weevers [1924] who employed plants with variegated leaves. In all, some twelve different species were investigated. In every case, save two (*Aesculus Hippocastanum* and *Cornus sanguinea*), both hexoses and sucrose were found in the green parts and only sucrose in the variegated portions. In *A. Hippocastanum* and *C. sanguinea* the variegated parts also contained a small amount of hexose. Since the variegated portions of these plants contain no chlorophyll and photosynthesis is impossible in these regions, it would seem a significant fact that no reducing sugars were found in these parts. The evidence from this fact alone is not conclusive, but in a second experiment, Weevers found that the leaves of the variegated form of *Pelargonium zonale* when placed in the dark for 48 hours were completely deprived of all sugars. At the end of this period the plants were allowed to assimilate, and it was discovered that the first sugar produced was a hexose and not sucrose.

A re-investigation of the sugars in the mangold has been carried out in America by Tottingham and his co-workers [1926]. The sucrose content of the leaves was found to vary very irregularly and over a considerable portion of the experimental period closely followed the course of the reducing sugars. Occasionally, however, it varied inversely as the hexose values. In view of these results, they considered that hexose and not sucrose was the first sugar of carbon assimilation.

At the conclusion of our own work an important paper on this subject appeared in America by Clements [1930]. Like ourselves (see below) he measured the fluctuations in the sugar fractions at hourly intervals in the leaves and petioles of *Helianthus annuus*, *Soja max* and the potato over 24-hour periods, and considered from the fact that the sucrose maxima were only reached after a long period of assimilation that simple sugars (pentoses and hexoses) and not sucrose are the first-formed carbohydrates in the leaf.

From the highly conflicting views that have been expressed in the literature on the nature of the first sugar of carbon assimilation, it was thought interesting to re-investigate the question and the results are recorded below.

Experimental.

The plant employed throughout this investigation was the daffodil, *Narcissus Pseudo-Narcissus* L. (vars. Emperor and Sir Watkin). It was decided to use

a monocotyledonous rather than a dicotyledonous plant to obviate the complications introduced by the presence of starch. The *Narcissus* leaf, like the greater number of monocotyledonous leaves, only forms hexoses and sucrose and contains but a negligible amount of starch in the guard-cells of the stomata. Further points in favour of using a monocotyledonous plant, such as *Narcissus*, are firstly, the high percentage of sugars in the leaf, and secondly, the plants are available for use in the early part of the year.

The experimental work was carried out at the Chelsea Physic Garden and the Royal Botanic Gardens, Regent's Park. The bulbs were specially selected and planted out in rows 18 in. apart and the plants were placed at a distance of 12 in. from each other.

Preparation of leaf material.

One of the main problems confronting us was to devise a satisfactory method of killing the leaves. Brown and Morris killed their material by placing it in an oven at 80°. But even with the leaves of *Tropaeolum*, which possess very thin laminas, maltose was found to be present. This maltose was subsequently shown by Davis, Daish and Sawyer to be due to diastatic activity at the high temperature employed and was not normally present in leaves. Parkin, on the other hand, found that little inversion occurred in the leaves of *Galanthus nivalis* in similar circumstances. This method of killing, however, is clearly inadmissible in the case of *Narcissus* leaves. The leaves are very thick and sappy and it is on this account unlikely that placing them in a temperature of 80° or 100° will bring about instantaneous death without inversion of sucrose. We therefore had to resort at first to the method used by Davis, Daish and Sawyer with certain slight modifications.

The leaves were first frozen at − 16° in a mixture of salt and ice for 6 hours. They were then ground with sand and the temperature was never allowed to rise to more than − 3°. The pulverised mass was thrown into boiling 95 % alcohol containing a little ammonia to neutralise organic acids and to stop enzymic activity. After boiling for half an hour, the leaves were extracted in a Soxhlet apparatus for 4–6 hours with the same alcohol used for killing. It was found that the preliminary grinding with sand rendered unnecessary the long extraction period (12–18 hours) used by Davis, Daish and Sawyer. The sugars were completely removed from the leaves in 4–6 hours. This method very unfortunately suffers from the defect that appreciable amounts of formaldehyde and acetaldehyde are produced. At the end of the extraction period, the solution showed a marked acid reaction, and, when the alcohol had been distilled off under reduced pressure and the residue treated with lead acetate to remove colouring matter and protein, it was found that both distillate and sugar solution contained aldehydes.

In the preliminary work of this investigation, commercial 95 % alcohol was used for killing the leaf material. After a time it was found that the alcohol residues obtained from the distillation of the leaf extracts possessed a powerful

smell of formaldehyde. The presence of formaldehyde was confirmed with Schiff's test and dimedon. To test further whether *Narcissus* leaves were peculiar in this respect of producing aldehydes other leaves were used, *e.g. Hedera helix*, *Abutilon* and *Pelargonium zonale*. Positive reactions were obtained in all cases and the reaction was particularly marked with *Hedera helix*. It is possible that the chlorophyll of the leaves oxidises the alcohol partly to aldehyde. In a second series of experiments absolute alcohol was diluted to 95 % and the same procedure as above carried out. In this case no formaldehyde was formed, but a considerable amount of acetaldehyde was obtained. The presence of either aldehyde will vitiate the hexose values obtained by copper reduction methods. To test this point we took samples of leaves and treated them with the alcohol method and compared the figures with those obtained by the method which we ultimately used in this investigation (see below). The figures given are representative of a large number of analyses which we carried out.

100 g. of leaves taken, chopped fine and mixed. 50 g. samples used for each test. Results expressed as a percentage of "residual dry weight."

Sample A. (Killed in alcohol.)		
	Hexose 18·3 %.	Sucrose 4·4 %.
Sample B. (Killed in boiling water.)		
	Hexose 15·5 %.	Sucrose 4·7 %.
Difference	Hexose 2·8 %.	Sucrose 0·3 %.

It will be seen that, although the sucrose value was almost unaffected, the hexose differed by 2·8 % in amount. This large difference in the values given for the hexose sugars was found in every case.

To discover whether the presence of aldehyde affected the optical rotation of sugar solutions, a 500 cc. sample of sucrose, fructose, and glucose was made up which gave a rotation of $\alpha_D = + 26'$. The solution was now evaporated under reduced pressure at 50° to 200 cc. and then made up to 500 cc. once more. The rotation was again $\alpha_D = + 26'$. A similar test was carried out after the addition of a small amount of acetaldehyde. Before evaporation the rotation was $\alpha_D = + 29', 3''$. After evaporation under reduced pressure and making the volume up to 500 cc., rotation $\alpha_D = + 36', 0''$. The presence of the aldehyde therefore also affects the polarimetric readings. Thus the figures obtained by Davis, Daish and Sawyer for total hexoses and also for glucose and fructose based on a copper reduction method and optical rotation must be looked upon with suspicion.

The following method was found to overcome this difficulty. The leaves were first frozen in the manner described above, ground up with sand and thrown in small quantities at a time into boiling water containing ammonia. They were allowed to boil gently for 10 minutes and then filtered through muslin and carefully squeezed. The squeezed mass was allowed to remain in cold water for 2 hours (cf. Parkin) and the process of filtering and squeezing was repeated. Four such treatments were found to remove completely all the sugars of the

leaf. The filtrates were mixed and distilled under reduced pressure to about 200 cc. bulk. 5 cc. of lead acetate solution were now added to clear the solution. An excess of lead acetate is to be avoided as it tends to destroy fructose. With this amount of lead acetate only a minimum quantity of lead remained in the solution and this was removed with sodium phosphate. The liquid was filtered off and washed free from the precipitate and made up to 500 cc.

Analysis of sugars.

Total hexose. For the analysis of total hexose and sucrose, Benedict's solution was employed. The method is susceptible of great accuracy and with the modifications we were able to introduce is very rapid, so that a number of determinations can be carried out in a short time. Although the white precipitate of copper thiocyanate gives a clearer end-point than Fehling's solution, it was still found difficult to see when the last trace of blue had disappeared from the Benedict's mixture. It was found, however, that if 20 cc. of a saturated solution of potassium thiocyanate containing 20 % of sodium carbonate were added to the Benedict's solution, this precipitate was removed and the end-point became very sharp. The addition of the excess of thiocyanate in no way interferes with the accuracy of the method as the following figures obtained with known sugar solutions show:

Glucose,	0·1 %	solution.	Found 0·1 %.
,,	3·0	,,	,, 2·99 %.
Sucrose,	0·1	,,	,, 0·099 %.
,,	2·0	,,	,, 1·996 %.

By carrying out the following procedure the analysis was greatly expedited and as many as thirty or forty estimations could be effected in the course of a day. 25 cc. of Benedict's solution were pipetted into a conical flask and 20 cc. of the potassium thiocyanate solution added. The mixture was raised to the boiling-point and 10 cc. of the sugar solution were run in. When the whole was gently boiling, it was back-titrated with a 0·5 % solution of glucose (25 cc. of Benedict ≡ 10 cc. of 0·5 % glucose sol.).

Sucrose. The sucrose was inverted in a separate portion with 10 % citric acid and the process described above repeated. Invertase was first employed for the inversion, but the citric acid and invertase values agreed so well that the citric acid was always employed and had the added advantage of rapidity.

Glucose and fructose. Attempts were made to determine the amounts of glucose and fructose in the leaf preparations. The iodimetric method of MacLeod and Robison [1929] was first utilised. Although the method yielded admirable results in our hands with mixtures of pure glucose and fructose in distilled water, it gave very discordant results with preparations from leaf material. The presence of the slightest trace of lead at once vitiated the method and even after all the lead had been removed with hydrogen sulphide and the solution subsequently aerated for 60 hours to remove all trace of the gas, discordant results were still obtained owing to the presence of traces of hydrogen

sulphide. The removal of the lead with sodium carbonate or oxalate is inadmissible with this method. Polarimetric methods were also abandoned as discordant results were again obtained. It was therefore decided to estimate only total hexose and the values recorded for reducing sugars include both glucose and fructose. No pentoses were found in our preparations.

Expression of results.

Previous workers have expressed their results as a percentage of the total dry weight of the leaf material (*i.e.* material dried to constant weight at 100°). The dry weight of a leaf, however, depends on two separate factors: the material already present and the sugars formed in assimilation. Dry weight does not therefore give a constant basis for the calculation of results, as the sugar values will vary with the rates of assimilation, respiration and translocation. As Mason and Maskell [1928] have pointed out in their work on translocation in the cotton plant, "residual dry weight," *i.e.* dry weight less total sugars, gives a better basis for the estimation of sugar percentages, since presumably residual dry weight will be a constant value over a short period of time. Chibnall [1922] has suggested that wet weight would present a more constant basis for the calculation of fluctuations in leaf products, since the variation in the water content of the leaf, relative to the total water present, is very small indeed. Further, the leaf tends to lose water and gain carbohydrates by day and to gain water and lose carbohydrates by night, on this account the total variation in water content will be, in general, less than that of dry weight. Nevertheless, there is little to show what this variation really is. Throughout this work, except where otherwise stated, we have calculated our sugar values as a percentage of the residual dry weight.

Method of experiment.

Errors due to sampling were small (1–2 %). In the field the leaves were chosen at random, one leaf from each plant, chopped fine and mixed. Samples were withdrawn for dry weight estimations and 50 g. samples were taken and accurately weighed for determinations of sugars. Continuous records were made of temperature and humidity, and stomatal aperture was measured by a Knight's porometer.

Previous workers have always determined their sugar values at comparatively long intervals of time, but it was considered advisable to estimate sugars at hourly intervals.

Before an experimental run, the plants were approximately graded as to height and then artificially darkened for periods that varied between 72 and 24 hours. All determinations were submitted to statistical treatment by the methods given by Fisher [1925].

The experimental work to be discussed falls under two heads.

(1) Sugar fluctuations before the flower buds had opened.

(2) Sugar fluctuations after the flower buds had opened.

Exp. 1. (*Chelsea Physic Garden.*) *March 31, 1930.*

The flower buds and stalks were removed, the plants were graded as to height and then darkened (March 28) at 10 a.m. The experimental run was begun at 10 a.m., March 31. The day was sunny at the beginning, but later clouded over and there were several heavy showers of rain. No porometer readings were taken on this occasion. Determinations of sugars were made at hourly intervals.

Table I.

	Time	Hexose %	Sucrose %	Total sugars %
Friday, Mar. 28, 1930	10 a.m.	2·65	7·80	10·45
Monday, Mar. 31, 1930	10 a.m.	6·24	4·1	10·31
	11 a.m.	5·30	5·8	11·10
	12 noon	5·40	5·2	10·60
	2 p.m.	6·80	8·5	15·30
	3 p.m.	3·90	6·6	10·50
	4 p.m.	4·80	11·7	16·50
	5 p.m.	3·60	7·7	11·30

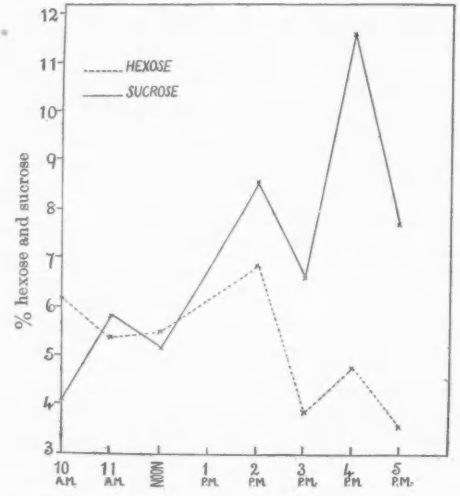

Fig. 1.

The results are graphically shown in Fig. 1. At the commencement of the run, the hexose was in excess of sucrose and at the end of the first hour, the former had fallen, while the latter showed a rise in concentration and the values of hexose and sucrose were approximately the same at the end of the second hour. At 2 p.m. both hexose and sucrose showed a sharp rise and thereafter the sucrose was always in excess of the hexose. It is a significant fact that the sucrose values only gradually reach a maximum, and the highest peak is finally attained after 6 hours of assimilation (4 p.m.). Between 12 noon and

1 p.m. there was a heavy shower of rain. Presumably during this period assimilation would be at a minimum (see below) and yet the sucrose values showed a continuous rise. Two maxima are shown on the curves: the first at 2 p.m. when the hexose value was 6·8 % and the sucrose 8·5 %, and the second at 4 p.m. when the hexose was 4·8 % and the sucrose 11·7 %. After each of these maximum points had been attained there was a fall in the concentration of both hexose and sucrose.

This experimental run, while giving no definite evidence for hexose as the first sugar of the leaf, nevertheless confirms the observations of Parkin that sucrose is in excess of hexose before the flowering period. It also shows that it is improbable that sucrose is the first sugar of photosynthesis, as the maximum sucrose values are only reached after a number of hours of assimilation and lastly, the rise in sucrose concentration is unaffected by rain.

Exp. 2. (*Royal Botanic Gardens, Regent's Park.*) *May 8, 1930.*

In Exp. 1 the sugar fluctuations were followed from a period of bright sunshine to late evening after the plants had been in darkness for 72 hours. In this second run, the plants were first allowed to flower and then flowers and flower stalks were removed before the determinations were made on the leaves. Analysis of the flower stalks showed that sucrose was in excess of hexose, whereas in the corollas, which contain no chlorophyll, only a trace of reducing sugars was found.

It was decided to darken the plants for 24 hours instead of for a longer time. The plants were again approximately graded as to height and then darkened. Continuous records of temperature and stomatal aperture were made. The experimental run began at 10 a.m. and was discontinued at 2 p.m. All samples were taken at hourly intervals.

Table II.

Thursday, May 8, 1930

Time*	Hexose %	Sucrose %	Total sugars %
10 a.m.	16·40	6·20	22·40
11 a.m.	17·50	5·30	22·80
Noon	11·80	7·50	19·30
1 p.m.	12·10	9·20	21·30
2 p.m.	19·80	11·70	31·50

* Summer time.

Fig. 2 shows the values for hexose, sucrose, temperature and stomatal aperture graphically expressed. The sugar percentages show that once the flowers had opened, hexose was in excess of sucrose in the leaves, a result in agreement with Parkin and also with other workers. During the first hour there was an increase in hexose and a fall in sucrose. If a hexose be the first sugar of assimilation, this result is to be expected. From 11 a.m., onwards to 2 p.m., there was a continuous rise in sucrose. The hexose, on the other hand, fluctuated markedly. At 11 a.m. there was a particularly heavy shower of rain and decrease in the light intensity, and during this time the hexose value

dropped by nearly 6 %. The sucrose, on the contrary, was not affected and rose steadily throughout this rainy period. Again, if a hexose be the first sugar of assimilation this result is to be expected. A curious point was noticed here, that the stomata of *Narcissus* are relatively insensitive to shock for they

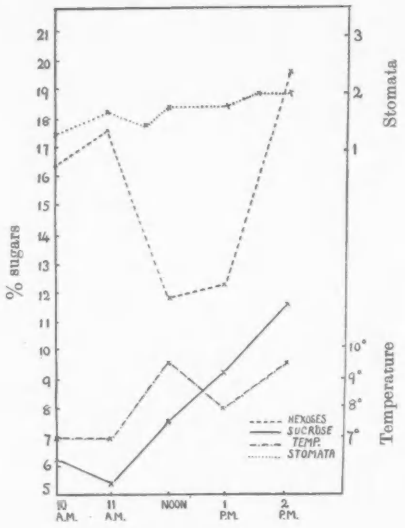

Fig. 2.

only slightly closed during the rain, which beat down with considerable violence.

Although this experiment yields very definite evidence that a hexose is the first sugar of assimilation, it was considered that a run extending from early dawn to late evening would yield results of still greater significance.

Neither Exp. 1 nor Exp. 2 was extended over a sufficiently long period of hours to allow of any correlation coefficients being calculated that would be statistically significant. It was also thought possible that in a run extending over a large number of hours, it might be possible to see whether there were any lag periods between hexose increase and sucrose increase and *vice versa*.

Exp. 3. (Royal Botanic Gardens, Regent's Park.) May 20, 1930.

All plants were graded as to height on the day before the experimental run. The flowers and flower stalks were then removed and the plants were artificially darkened at 10 a.m. The coverings were removed at 10 p.m. on the same evening and the determinations were commenced at 3.45 a.m. on the following morning before daylight. Continuous records were made of humidity, temperature and stomatal aperture.

Table III.

Tuesday, May 20, 1930

Time*	Hexose %	Sucrose %	Total sugars %
3.45 a.m.	18·20	5·30	23·50
4.45 a.m.	16·70	6·50	23·20
5.45 a.m.	17·00	7·02	24·02
6.45 a.m.	18·70	7·06	25·76
7.45 a.m.	17·50	5·50	23·00
8.50 a.m.	18·10	7·10	25·20
9.50 a.m.	16·60	6·90	23·50
10.45 a.m.	19·10	6·10	25·20
Noon	18·90	7·80	26·70
12.55 p.m.	15·30	9·20	24·50
1.50 p.m.	17·80	12·40	30·20
2.45 p.m.	19·50	11·40	30·90
3.30 p.m.	18·00	10·00	28·00
4.30 p.m.	14·10	11·70	25·80
5.45 p.m.	13·70	10·80	23·50
6.45 p.m.	13·30	12·20	25·50
7.45 p.m.	12·90	13·40	26·30
8.45 p.m.	14·10	13·40	27·50
9.40 p.m.	11·80	10·40	22·20

(Results calculated as percentage of residual dry weight.)

Table IV.

Tuesday, May 20, 1930

Time*	Hexose %	Sucrose %	Total sugars %
3.45 a.m.	1·700	0·522	2·222
4.45 a.m.	1·600	0·664	2·264
5.45 a.m.	1·550	0·640	2·190
6.45 a.m.	1·700	0·640	2·340
7.45 a.m.	1·800	0·576	2·376
8.50 a.m.	1·700	0·712	2·412
9.50 a.m.	1·700	0·712	2·412
10.45 a.m.	1·800	0·570	2·370
Noon	1·750	0·712	2·462
12.55 p.m	1·500	0·900	2·400
1.50 p.m.	1·500	1·150	2·650
2.45 p.m.	1·700	0·998	2·698
3.30 p.m.	1·800	0·998	2·798
4.30 p.m.	1·550	1·282	2·832
5.45 p.m.	1·550	1·234	2·784
6.45 p.m.	1·400	1·282	2·682
7.45 p.m.	1·350	1·378	2·728
8.45 p.m.	1·350	1·282	2·632
9.40 p.m.	1·300	1·140	2·440

(Results calculated as a percentage of wet weight.)

* Summer time.

Figs. 3 and 4 show these results graphically expressed for the values calculated on residual dry weight and wet weight respectively together with the curve for stomatal aperture.

The correlation coefficient (r) for hexose and sucrose was $r = 0{\cdot}71 \pm 0{\cdot}12$. This value is statistically significant and shows that there is a direct correlation between these two classes of carbohydrates. The correlation between hexose and temperature was $r = 0{\cdot}47 \pm 0{\cdot}18$, whereas between sucrose and temperature the value was $r = 0{\cdot}3 \pm 0{\cdot}02$. The latter value is not statistically significant, the former barely so. Nevertheless, there is a closer correlation between hexose

and temperature than between sucrose and temperature. There was no correlation between humidity and dry weight over the total experimental time (19 hours), $r = 0{\cdot}35 \pm 0{\cdot}206$, nor between 3.45 a.m. and 2.45 p.m.,

$$r = 0{\cdot}331 \pm 0{\cdot}26,$$

but there was a significant correlation between these values for the period between 2.45 p.m., and 9.40 p.m., $r = 0{\cdot}8839 \pm 0{\cdot}089$ (see Fig. 7).

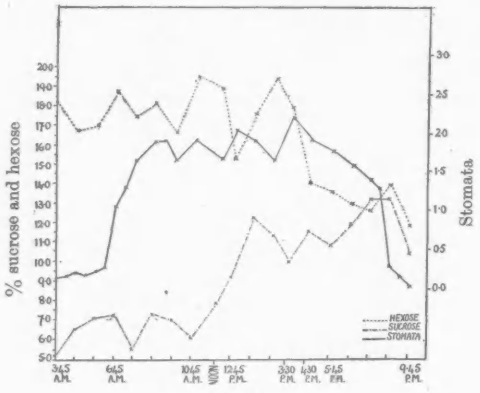

Fig. 3.

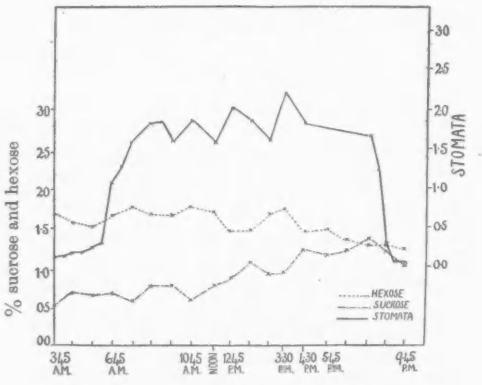

Fig. 4.

An examination of Fig. 4 shows a marked fluctuation in the hexose values, whereas those of sucrose only begin to show a continuous rise after 10.45 a.m. There is a sharp rise in the hexose value at 9.50 a.m. which is followed by a fall in the sucrose concentration and there is a lag period of 1 hour before the sucrose begins to mount once more. We have already observed that there is a correlation between the hexose and sucrose values ($r = 0{\cdot}71 \pm 0{\cdot}12$) and

that there is a lag period of 1 hour between increase in hexose and increase in sucrose (9.45 a.m. and 10.45 a.m.). We should therefore obtain a higher correlation between hexose and sucrose if the value for sucrose be shifted forward a period (4 hours). The result of making this shift was to increase r from $0{\cdot}71 \pm 0{\cdot}12$ to $0{\cdot}77 \pm 0{\cdot}106$. Variations in hexose are therefore reflected in sucrose and the lag period is significant. Rain at 10.45 a.m. led to a fall in the hexose, but the sucrose still showed a rise. Between 1.50 p.m. and 3.30 p.m. there was an increase in hexose, whereas after a lag period of 1 hour the sucrose fell. The maximum hexose percentage was obtained at 2.45 p.m., and thereafter there was a continuous fall. The sucrose, on the other hand, was still increasing in amount till the maximum percentage was reached at 8.45 p.m. when it crossed the hexose curve, after which it fell away. During the early hours of the morning, 4.45 a.m. to 6.45 a.m., when the stomata were actively opening, the hexose was increasing in amount and the sucrose decreasing.

Consideration of Fig. 4 based on wet weight follows in general outline the results obtained with residual dry weight, although the fluctuations are not so marked for either hexose or sucrose. Nevertheless, an examination of the hexose curve and stomatal aperture shows that when the stomata were actively opening there was increase in the hexose, while the sucrose remained relatively constant. Again, the rise in sucrose between 7.45 a.m. to 8.45 a.m. shows a marked lag behind the increase in hexose. As in Fig. 3, there is a continuous increase in sucrose after 10.45 a.m. and a corresponding fall in hexose, and the continuous fall in hexose after 3.30 p.m. is accompanied by a continuous increase in sucrose to a maximum at 7.45 p.m. The stomata started to close at 5.45 p.m. and the hexose values had begun to fall an hour before this period, whereas the sucrose still continued to rise.

Examination of the hexose and sucrose curves, based on either residual dry weight or wet weight shows that there is a continuous negative drift with time in the hexose values ($r = -0{\cdot}84$) through the day and a continuous positive drift in the sucrose concentrations ($r = +0{\cdot}875$). Parkin in one of his experiments found the same result, but interpreted it as showing that sucrose was the first sugar of assimilation. On the assumption that hexose and not sucrose is the first sugar and that an equilibrium condition exists between them this result is to be expected.

The hexose values, based on residual dry weight show three maxima, the first at 6.45 a.m. (18·7 %), the second at 10.45 a.m. (19·1 %) and the third at 2.45 p.m. (19·5 %). After each of these maxima has been attained, the sucrose values show a continuous rise for periods varying between 4 and 5 hours. It would therefore appear that the hexose reaches a certain definite concentration and then suffers condensation to sucrose.

The curves for total sugars, based on residual dry weight and wet weight respectively, show a very considerable shift in the maximum position. In the case of the residual dry weight curve (Fig. 5) the maximum total sugars are found at 2.45 p.m., whereas with wet weight (Fig. 6) the maximum is not

attained till 4.30 p.m. This result is no doubt due to the marked correlation between dry weight and humidity that was found to exist after 2.45 p.m.

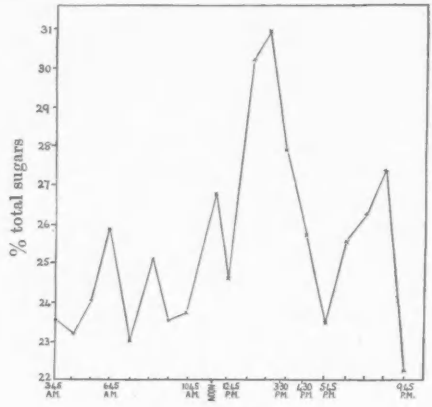

Fig. 5.

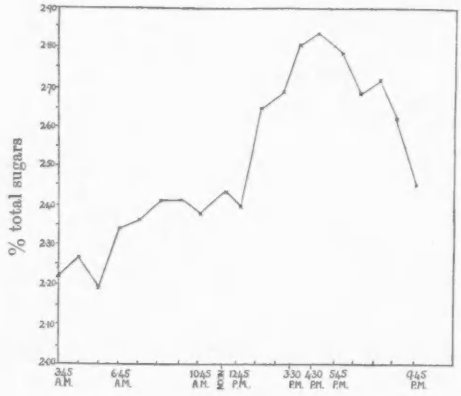

Fig. 6.

Discussion.

The utilisation of a leaf such as that of *Narcissus*, in an investigation of this nature, reduces the problem of the first sugar of assimilation to its simplest proportions and the complications introduced by the presence of starch are eliminated. Both Brown and Morris, who worked on a dicotyledonous leaf, and Parkin, who used a monocotyledon, claimed that sucrose was the first sugar and that it was inverted to glucose and fructose for translocation to the storage organs. The value of this work, apart from errors of technique in the case of Brown and Morris's estimations, is largely diminished by the fact that the leaf

sugars were only determined at long intervals of time. Again, the figures obtained by Davis, Daish and Sawyer are vitiated by faulty technique as far as the reducing sugars are concerned. But even if this be allowed for, an examination of their curves gives no conclusive evidence for the production of sucrose as the first sugar of photosynthesis. In fact the curves obtained for the October experiment show very marked lags between the hexose and sucrose, and the latter lags behind the former.

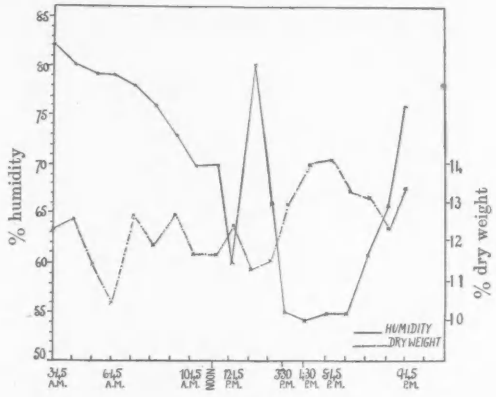

Fig. 7.

The claim that carbohydrates are translocated to the storage organs as glucose and fructose has not been substantiated by the recent work of Mason and Maskell on carbohydrate transport in the cotton plant. Here the sugars leave the leaf as sucrose. It is possible so far as the values recorded by Davis, Daish and Sawyer are concerned that inversion of sucrose had taken place in the petioles due to the method of killing and that it was on this account that the hexose values exceeded the sucrose percentages. The petioles of the mangold are, relatively to the leaf lamina, comparatively massive organs and it is probable that they were not instantaneously killed and that for a short time, owing to high temperature, invertase activity was high.

Clements [1930], who worked with three different species and, like ourselves, estimated sugar variations hourly, considered that his data supported the view that simple sugars (pentoses and hexoses) precede the formation of sucrose in the leaf and that the latter subserves the function of a temporary storage product and is also the mobile form of sugar in the plant. We are in agreement with the general interpretations of this worker. It is unfortunate, however, that he should have employed the alcohol method of killing the leaves. The error introduced by the presence of aldehyde is very material. Moreover, the question whether pentoses be a direct product of photosynthesis is doubtful; we were quite unable to find any pentoses in our preparations. He also puts forward the claim that hemicelluloses show fluctuating values in the

leaf and form an important storage product. The method of estimating hemicellulose was to hydrolyse the leaf residue with 2·5 % sulphuric acid. This method is not advisable, since not only will hemicelluloses suffer hydrolysis, but so will pentosans and possibly the cellulose of the leaf cells. Clements does not define the term "hemicellulose" and it is impossible to say what value he attaches to it, and certainly his analytical procedure allows of little weight being attached to his figures for these bodies. It is also unfortunate that he should have calculated his results on a wet weight basis and not as a percentage of the residual dry weight, and lastly no attempt was made to submit the data to statistical treatment.

The evidence presented in the present investigation can leave little doubt that a hexose precedes the formation of sucrose in the green leaf. There is in the first place a direct correlation between these two classes of sugar. There is also a greater correlation between temperature and hexose than between temperature and sucrose. It is true that the former correlation is barely statistically significant, but on the other hand, plants such as *Narcissus* and other bulbous monocotyledons are in active growth in the cold seasons of the year and it is therefore possible that temperature does not play as great a part in their metabolic activities as in summer vegetation. There is a significant lag between increase in hexose and increase in sucrose, and even in the case in which sucrose was in excess of hexose in the leaf, *i.e.* before flowering, the sucrose values only slowly drifted towards a maximum value.

The investigations of F. F. Blackman and Matthaei [1905] on the assimilation rate of *Helianthus annuus* under natural conditions showed that there was a marked fall in the assimilation rate during rain. This condition is typified as far as our hexose curves are concerned. In every case at the onset of rain the hexose fell and the sucrose was unaffected and showed a continuous rise. Lastly, there is a continuous negative drift of hexose from the beginning of photosynthesis to darkness, and a positive drift of sucrose during the same time —a result to be expected if the hexose be continuously suffering condensation to sucrose.

If the synthesis of sugars in the leaf be looked upon as an equilibrium reaction between hexose and sucrose:

$$\text{Hexose} \rightleftharpoons \text{Sucrose},$$

and if it be further supposed that when the hexose reaches a certain concentration there is condensation to sucrose, or if it fall below a certain value the sucrose is inverted to hexose, then this result is also paralleled in our curves. In Exp. 3, three maxima were found for the hexose values and after each there was a continuous rise in the concentration of sucrose. Priestley [1924] has taken much the same view for the synthesis of sugars in the leaf. He considered that since sugars are formed as intermediate steps in the formation of more complex anhydrides, such as starch, it is necessary that these reactions should take place smoothly and rapidly.

With regard to the function of sucrose in the plant, Priestley has put forward an unusual suggestion and one for which we can find no experimental support, namely, that sucrose is a by-product of ageing protoplasm in meristematic cells and that as these cells become vacuolated sucrose is released. It would thus appear from this view that sucrose at the growing meristems of the plant arises as a catabolic product. As Clements has pointed out, this view at once eliminates the places of storage, as, for example, in the sugar cane and sugar beet, and also eliminates the leaves, for there is a considerable amount of sucrose present in these parts. It may well be asked how the sucrose is formed in these regions.

The argument advanced by Davis, Daish and Sawyer for sucrose as the first sugar, based on the fact of the well-known enol conversion of glucose to fructose and mannose and the absence of mannose from leaves, does not apply here. The enolic form of glucose can only exist in alkaline solution, whereas the reactions in the leaf are taking place in acid media.

If a hexose be the first formed product of photosynthesis, a further difficulty has to be contended with, for not one, but two hexoses, glucose and fructose, must be formed to give cane sugar by condensation. In this connection, Nef [1913] showed that aqueous solutions of *d*-glucose, *d*-fructose and *d*-mannose, in the presence of a 0·05 equivalent of calcium hydroxide at the ordinary temperature, gave mixtures composed of *d*-mannose, *d*-glucose, *d*-fructose, *d*-*pseudo*fructose, as well as *α*-, *β*- and *d*-glutose. Similar results have been obtained by Wilbur and Spoehr, who have been able to bring about interconversion of sugars in the presence of solutions of disodium hydrogen phosphate and in neutral mixtures of this salt and sodium dihydrogen phosphate. Again, in the animal economy, fructose is converted into glucose. On these grounds, if glucose be first produced in the leaf, the mesophyll must be possessed of a special and at present unknown mechanism to convert part of this glucose to fructose and other hexoses. Nevertheless, mannose as such has never up to the present been isolated from the leaf. Its recognition should be simple as it is the only known hexose that forms a sparingly soluble hydrazone.

The analytical data presented in this paper show that a hexose is the sugar first formed in the leaf in photosynthesis. We would therefore suggest that glucose is the first sugar formed and that part of this is converted into fructose. Condensation now takes place to sucrose and it is as sucrose that translocation takes place to the bulbs. During the early growth period of the plant, up to the formation and opening of the flower, an excess of sucrose is present in the leaves and masks the hexose, possibly owing to the high needs for sucrose in flower formation. Sugar is translocated to the flower as sucrose. It will be remembered that we always found sucrose to be in excess of hexose in the flower stalk and only a trace of hexose was present in the corolla, a region in which no photosynthesis can take place. At the termination of the reproductive phase, large amounts of carbohydrate will be needed for the dormant period of the plant and growth in the subsequent season. At this stage hexose

exceeds sucrose in the leaves, perhaps owing to the greater amount of assimilation at this time.

SUMMARY.

An investigation has been made of the carbohydrates in the leaf of the daffodil, *Narcissus Pseudo-Narcissus* L. Only hexoses and sucrose were found to be present. Evidence is presented to show that the first sugar of photosynthesis is a hexose and not sucrose.

We are deeply indebted to Mr W. Hales of the Chelsea Physic Garden, to the Authorities of the Royal Botanic Gardens, Regent's Park and to Mr Weaver (Curator) for their help and kindness in growing the material for this investigation and for allowing us to use the gardens at times which must have been highly inconvenient to themselves. We gratefully acknowledge the help given us by Mr J. G. Boswell in numerous directions. We should also like to record our indebtedness to Mr C. S. Semmens for help in preparation of much of the material.

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CXXXVII. THE CHEMISTRY OF THE WHITE ROTS OF WOOD.

I. THE EFFECT ON WOOD SUBSTANCE OF *POLYSTICTUS VERSICOLOR* (LINN.) FR.

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Introduction.

The somewhat arbitrary classification of the wood decays caused by fungi into "brown" and "white" rots has in recent years been the subject of controversy, as it has been suggested that more accurate classification can be based on strictly chemical evidence regarding the effects of the various wood-rotting fungi on wood substance.

So far as "brown" rots are concerned a sufficiently representative number of these would appear to have been studied in order to make it possible, within the limits of available knowledge, to assign a hitherto unstudied fungus to this class on chemical grounds alone, for it has been shown that the chemical effects of "brown" rots on wood substance can strictly be compared to those of simple aqueous acid hydrolysis [Campbell and Booth, 1929]. The specificity of fungi generally with regard to time, mode and position of attack, together with the important question of host selection, has been postulated as a cogent factor in support of the view that the ultimate chemical effects of fungi on wood may be so variable as to preclude the accurate classification of decay into types on chemical grounds. Having in mind, however, the general composition of the skeletal substance of wood and its comparative chemical uniformity throughout the wide range of hardwoods and softwoods for which analytical data have been obtained, it is conceivable that, apart from climatic and other external conditions, the selective capacity of wood-rotting fungi must be controlled largely by the presence or absence in specific woods of substances, other than wood substance proper, which are toxic or otherwise intolerable to certain fungi. This is borne out by the work of Hawley, Fleck and Richards [1924], and of Sowder [1929], and is generally accepted as the explanation of the immunity to decay of notably durable woods. In this connection attention is directed to the conclusions arrived at by Rege [1927] and more recently by Norman [1929, 1] in their estimates regarding the "decomposability" of cellulosic

materials. The decomposability of woods cannot be predicted by any scheme such as those proposed by the above authors, since any prediction as to susceptibility to decay which fails to take into account the nature of the extraneous components of a specific wood and the different types of decay to which wood is subject would in the light of abundant evidence prove to be useless. None of the woods mentioned by Norman [1929, 1] can be regarded as in any sense particularly resistant to fungal decay. The decomposability of wood even when considered quite apart from extractives cannot be predicted by any fixed scheme, for the factors governing the susceptibility of its principal components are as yet but little understood. In certain quarters the presence of lignin in wood is regarded as a factor inhibitory to decay, but this can only hold for fungi of the brown-rot type, and when it is realised that some fungi actually bring about the decomposition of lignin, this belief must unconditionally be abandoned.

It seems certain, however, that the polysaccharides of wood are a potential source of food for fungi, and equally certain that all fungi which attack carbohydrates in preference to lignin do so in the same manner, namely, by hydrolysis of insoluble polysaccharides to soluble monosaccharides. Thus, in so far as the ultimate effects on wood substance are concerned, the brown rots would seem to form a definite class, but since little of a conclusive character is known of the chemistry of the lignin in wood, much experimental work will be necessary before it will be possible with any degree of certainty to state whether this component is always attacked in the same manner by fungi of the "white" rot type.

The "oxidation" or "corrosion" theory regarding the mechanism of white rots has previously been referred to [Campbell and Booth, 1929], but sufficient stress has not been laid on the question as to whether white-rot fungi are capable of deriving nourishment solely from the lignin in wood, or indeed whether attack on the lignin complex is accompanied by simultaneous degradation of polysaccharides. The present investigation has been undertaken to ascertain the detailed effect of *Polystictus versicolor* (Linn.) Fr. in pure culture on wood substance, as it is believed that this fungus produces a white rot. The effect of the decay has been examined in samples of the wood of both beech and ash.

Experimental.

A sample of air-seasoned beech heartwood was ground to pass a 60- and be retained by an 80-mesh screen. After thorough mixing, a portion of the wood flour was removed for analysis (Table I).

A further portion of the original wood was now extracted with 1 % sodium hydroxide for 1 hour at 100° under the conditions laid down by Schorger [1926, p. 506], and, after filtering off the extract, washing with hot water and drying at 105° the residue was analysed (Table I).

Table I. *Analysis of original 60–80-mesh beech heartwood before and after alkali extraction.*

(Results expressed as percentages by weight of original oven-dry wood.)

	Original wood	After alkali extraction
Cold water-soluble ...	0·64	—
Hot water-soluble ...	1·65	—
1 % NaOH-soluble ...	16·63	—
Cellulose	57·49	58·67
Lignin	23·79	19·79
Total pentosans	26·95	19·02
Pentosans in cellulose ...	15·5	18·42
Pentosans not in cellulose	11·45	0·60
Methoxyl content ...	6·33	4·81

The effect of Polystictus versicolor (*Linn.*) *Fr. on beech wood.*

Portions of the wood sample were sterilised by steaming at 100° for 30 mins. on each of three successive days and then incubated with *P. versicolor* at 20°. At the end of 6 and 10 weeks respectively, samples were taken for analysis. The wood was found to be distinctly acid to litmus and was accordingly collected on a linen filter, washed with cold water till acid-free and dried and sterilised at 105°. The analyses of the decayed residues are given in Table II. The effect of 1 % sodium hydroxide on these residues was then determined as for the original wood (Table II).

Table II. *Analysis of 60–80-mesh beech heartwood after decay by* P. versicolor *and washing with cold water.*

(Results expressed as percentages by weight of the original oven-dry wood.)

	Decayed wood		Decayed wood after alkali extraction	
	(1)	(2)	(1)	(2)
Duration of decay ...	6 weeks	10 weeks	6 weeks	10 weeks
Loss due to decay ...	5·68	20·15	—	—
Cold water-soluble ...	1·58	0·71	—	—
Hot water-soluble ...	2·88	1·88	—	—
1 % NaOH-soluble ...	18·32	15·03	—	—
Cellulose	55·77	48·55	55·19	47·02
Lignin	21·05	16·91	16·31	·13·56
Total pentosans	22·16	18·36	17·77	15·35
Pentosans in cellulose ...	13·94	13·63	16·52	14·07
Pentosans not in cellulose	8·22	4·73	1·25	1·28
Methoxyl content ...	5·38	4·42	4·31	3·58

The effect of mild aqueous acid hydrolysis on the original beech wood.

100 g. of the original beech wood were now incorporated with 2 litres of 0·25 % aqueous hydrochloric acid and maintained at 100° for 80 mins. After removal of the acid and thorough washing with hot water the residue was dried at 105° and analysed (Table III).

Table III. *Analysis of original 60–80-mesh beech heartwood after hydrolysis with 0·25 % HCl at 100° for 80 mins.*

(Results expressed as percentages by weight of original oven-dry wood.)

Loss due to hydrolysis	5·5
1 % NaOH-soluble	23·81
Cellulose	52·66
Lignin	22·69
Total pentosans	22·10
Pentosans in cellulose	12·27
Pentosans not in cellulose	9·83
Methoxyl content	4·68

The effect of an acid-alcohol solution on the original beech wood.

100 g. of the original wood were incorporated with a solution consisting of 1 litre of 0·25 % aqueous hydrochloric acid and 1 litre of 97 % ethyl alcohol and maintained at 100° for 80 mins. The extract was filtered off and the residue was washed with ethyl alcohol, then with hot water until free from acid, dried at 105° and analysed (Table IV). The effect of 1 % sodium hydroxide on this residue was also determined (Table IV).

Table IV. *Analysis of original 60–80-mesh beech heartwood after treatment with one volume 0·25 % HCl, one volume 97 % alcohol at 100° for 80 mins.*

(Results expressed as percentages by weight of original oven-dry wood.)

	Residue	Residue after alkali extraction
Loss due to treatment ...	5·2	—
Cold water-soluble ...	0·40	—
Hot water-soluble ...	1·28	—
1 % NaOH-soluble ...	16·02	—
Cellulose	56·66	56·94
Lignin	20·96	17·72
Total pentosans	23·38	18·20
Pentosans in cellulose ...	15·14	17·45
Pentosans not in cellulose	8·24	0·75
Methoxyl content ...	5·27	4·77

The effect of P. versicolor (*Linn.*) *Fr. on ash heartwood.*

A sample of ash heartwood, the analysis of which is recorded in Table V, was divided into a series of small blocks each of which was inoculated with

Table V. *Analysis of 60–80-mesh ash heartwood before and after decay by* P. versicolor.

(Results expressed as percentages by weight of oven-dry material.)

	Original wood	Decayed wood
Loss due to decay ...	40·76	—
1 % NaOH-soluble ...	26·64	24·74
Cellulose	49·37	55·87
Lignin	22·11	22·90
Total pentosans	23·56	24·46
Pentosans in cellulose ...	11·98	14·19
Pentosans not in cellulose	11·58	9·27

P. versicolor after the usual sterilisation. Decay was allowed to proceed to an advanced stage. After removal of the surface mycelial growth, the blocks were dried at 105° and weighed. The decayed material was now ground to pass a 60- and be retained by an 80-mesh screen and, after thorough mixing, analysed in the usual manner (Table V).

Since the average loss in weight of the blocks was 40·76 % of their original dry weight this figure was employed in order to calculate the analytical data for the decayed wood as percentages of the oven-dry weight of the original wood (Table VI).

Table VI. *Analysis of 60–80-mesh ash heartwood decayed by* P. versicolor.

(Results expressed as percentages by weight of the original oven-dry wood.)

1 % NaOH-soluble	14·65
Cellulose	33·11
Lignin	13·26
Total pentosans	14·49
Pentosans in cellulose	8·41
Pentosans not in cellulose	5·98

Discussion.

Comparison of the data in Table I shows the effect of 1 % sodium hydroxide on the original wood. After alkali extraction the yield of cellulose as determined by the Cross and Bevan method is actually greater than that obtained from the original wood. The pentosan content of the cellulose is enhanced as a result of the alkali treatment, but at the same time it can be seen that part of the cellulose complex is soluble in alkali since the percentage increase in yield of this component is less than the increase in its pentosan content. This peculiar effect of alkali appears to be characteristic of hardwoods since similar results have been obtained in the case of oak [Campbell and Booth, 1930]. The results indicate that during the alkali treatment the furfuraldehyde-yielding complexes which are not associated with the cellulose in the original wood are involved in some change which results in the solution of one portion in the alkali and the association of another portion with the cellulose.

Various sources of error in the estimation of the pentosan content of wood by the phloroglucinol method have long been recognised. These have been discussed by Hawley and Wise [1926, p. 155] who have concluded that up till 1926 no entirely satisfactory method of estimating pentosans had been devised. Some of the known facts concerning the sources of error in the phloroglucinol method have recently been recapitulated by Norman [1929, 2], who has suggested certain corrections in view of the fact that, on distillation with 12 % hydrochloric acid, plant constituents other than pentosans yield appreciable amounts of furfuraldehyde as well as other bodies which are precipitated by phloroglucinol. Moreover, there is evidence in favour of the view that the furfuraldehyde phloroglucide precipitate has no fixed chemical composition

[Hawley and Wise, 1926, p. 155], and that extraction of this precipitate with alcohol does not appear to remove all the hydroxymethylfurfuraldehyde phloroglucide [Hawley and Wise, 1926, p. 157]. It would appear, therefore, that no modification of this method of determining pentosans can ever be universally accepted. Antoniani [1928] has suggested that hydroxymethylfurfuraldehyde can be removed from the hydrochloric acid distillate by redistillation, but again a correction is necessary for the diminution of furfuraldehyde which ensues. This author also concludes that the furfuraldehyde phloroglucide is not always of exactly similar composition.

It is therefore considered that in wood analysis the pentosan determination of Schorger [1926, p. 532] should remain in use until an acceptable substitute is forthcoming, since from the purely statistical standpoint this determination has been shown in previous work to be of unquestionable value.

Comparison of Tables I and II shows the effect of *P. versicolor* on the principal components of beech wood. It can be seen that even though the decayed residue had been washed with cold water prior to analysis it still retained more water-soluble material than the original sound wood. An increase in water-soluble material is a common result of fungal decay. The data further show that all of the remaining wood components have suffered depletion during decay, but that in the incipient stages depletion is most pronounced in the pentosans not in the cellulose. The extent of lignin depletion is relatively greater than that of the cellulose, and in the initial stages of decay the loss in this latter constituent is practically accounted for by the depletion of its pentosan content. The fall in the methoxyl content is small in comparison to that of the lignin content. Few data relative to this type of decay have hitherto been available, for, although an analysis has been made of apple wood infected with the same fungus [Hawley and Wise, 1926, p. 300], the absence of lignin determinations makes it impossible to define the type of decomposition. Thus in Tables II, V and VI data have been introduced to show that as the decay caused by *P. versicolor* becomes more advanced, decomposition of the cellulose proper takes place in addition to that of the other major wood components. The results in Table V reveal that in consequence of decay there is an apparent accumulation of cellulose, and a depletion of pentosans not in the cellulose, but it is seen from Table VI that accumulation of cellulose does not take place. On the contrary, the data reveal that of the total loss in weight of 40·76 % sustained as a result of decay, 16·26 % is accounted for by cellulose depletion and 8·85 % by lignin depletion. Further, it can be seen that in the advanced stages of decay the pentosans not in the cellulose are still depleted to a greater extent than the pentosans in the cellulose, but although these components are the first to be attacked by the fungus, the rate at which they are decomposed is diminished as decay proceeds, and preferential attack is made on the cellulose and lignin.

With regard to the alkali solubility of wood decayed by *P. versicolor*, comparison of Tables I and II reveals that in the initial stages of decay the alkali solubility of the residue is only slightly greater than that of the original wood.

Further examination of the data shows that the cellulose in partly decayed wood is more susceptible to alkali than the corresponding component in sound wood, but that after 10 weeks' decay the alkali solubility of this component of the decayed residue begins to decrease. The alkali solubility of lignin is practically the same in both sound and decayed wood, but the furfuraldehyde-yielding complexes in decayed wood are less soluble in alkali than the corresponding components in sound wood. By comparison with previous data [Campbell and Booth, 1929] it can be seen that where the losses in weight due to incipient decay are approximately the same, a "brown" rot is responsible for a much greater increase in alkali solubility of the wood residue than is the rot produced by the fungus under investigation. In this latter type of decay the total alkali solubility of the residual cell wall substance tends to decrease as decay becomes more advanced, the decrease in solubility of the furfuraldehyde-yielding complexes being greater than the total increase in solubility of cellulose and lignin. If it is conceded that the water-soluble material in wood is also soluble in alkali it can be seen that, if the total percentage of water-soluble material be subtracted from the percentage of alkali-soluble material in sound and decayed wood respectively, the resultant figure for the alkali solubility of the decayed wood is actually less than that for the original sound wood. In the advanced stages of decay (Tables V and VI) a large decrease in alkali solubility is obtained.

From the foregoing evidence it is apparent that the decay of wood caused by *P. versicolor* differs radically in its effects from those of the more widely studied class of "brown" rots.

Consideration of the ultimate chemical nature of the decay.

The production of acids in the course of the decay of wood by fungi raises the important question as to whether such acids are in the first instance secreted by the fungus as a direct means to effect decay, or whether they are merely formed during decay as decomposition products of wood substance. In the case of the brown rots, where decomposition has been shown to be strictly comparable in effect to mild aqueous acid hydrolysis [Campbell and Booth, 1929], it has not yet been shown experimentally whether the decay is attributable to a hydrolytic enzyme, an acid secreted by the fungus, or both of these agencies, but in the present study it is apparent that despite the observed acidity of the decayed wood, acid hydrolysis cannot solely be responsible for the decay. Comparison of the data in Tables II and III shows that where the respective losses in weight due to decay by *P. versicolor* and an acid hydrolysis are approximately the same, the compositions of the respective residues betray the characteristic differences in the effects of the two processes on wood substance.

None the less it is conceivable that the acid produced during decay may play some part in the decomposition of the wood substance, but its normal hydrolytic effect must be modified by some other agency. In seeking a means whereby

an acid hydrolysis of wood can be so modified as to result in lowered alkali solubility of the residue, marked attack on lignin, and depletion of pentosans not in the cellulose which is more intense than the depletion of pentosans in the cellulose, reference must be made to previous work [Campbell, 1929] in which it is shown that these results can be produced by the incorporation of an alcohol with the hydrolysing medium. A detailed study of the wood-acid-alcohol reaction has revealed the variations in effect obtainable on wood substance with different proportions of acid and alcohol, as well as when the duration and temperature of the reaction are altered. For comparative purposes therefore the effect of an acid-alcohol solution has been determined on the original beech wood used in this investigation and the data in Table IV show that, although the extent of decomposition obtained by this treatment was not so great as that produced in the sample of decayed wood, the order of the effect of incipient decay on wood substance is distinctly similar to that of the acid-alcohol solution. The lower alkali solubility in Table IV is undoubtedly explained by the fact that most of the water-soluble material has been washed out of the residue. With regard to the manner in which ash wood (Tables V and VI) has been decomposed by the same fungus it is interesting to note that when wood substance is subjected to an acid-alcohol solution under conditions calculated to produce a similar loss in weight [Campbell, 1929], the manner in which the principal wood components are decomposed, as evinced by the comparative compositions of original wood and residue, is similar in effect to the action of *P. versicolor*. It is thus conceivable that a closer understanding of the wood-acid-alcohol reaction and its ultimate mechanism may lead to a true chemical definition of this type of decay.

Experimental evidence at this stage does not warrant the conclusion that the decay ultimately consists in the simultaneous action of an acid and an alcohol on wood substance, for, although there is evidence that acid is produced during the decay, the manner in which an alcohol could be produced is not clear.

The view has been expressed by Wehmer [1927] that in white rots lignin is depleted by a process of oxidation. Much support would be gained for this theory if it could be shown experimentally that the wood-acid-alcohol reaction were similar in effect to a catalytic oxidation of wood substance in the presence of acid. Dorée and Cunningham [1913] have shown that acetic and formic acids are formed when moist wood is oxidised by ozonised oxygen, and, theoretically, it is not improbable that an oxidase secreted by a fungus might produce similar results. In such an event, further simultaneous action of oxidase and acids might complete the process of decomposition. Such a theory would appear to be compatible with experimental evidence, for it would explain the early attack of *P. versicolor* on the lignin and pentosans, and the later attack on the cellulose of wood substance. Acids might be formed by the action of an oxidase on lignin and pentosans, and then react together with the oxidase to bring about the later stages of the decay in which cellulose is decomposed.

SUMMARY.

1. The chemical effect of *P. versicolor* (Linn.) Fr. on the wood of beech and ash has been examined in detail.

2. The decay is characterised by the following salient features.

(*a*) The decayed wood is more soluble in water than the original sound wood.

(*b*) In the early stages of decay the residue is slightly more soluble in alkali than the original wood, but alkali solubility tends to decrease as decay becomes more advanced.

(*c*) The pentosans of wood substance and lignin are the first components to be attacked, and of these the pentosans not in the cellulose suffer most depletion.

(*d*) As decay becomes more advanced the rate at which pentosans are depleted is diminished, and preferential attack is made on the cellulose proper as well as on the lignin.

3. It is concluded that the fungus causes a white rot on the wood of beech and ash.

4. The effects of the decay on wood substance are closely comparable to those produced by acid-alcohol solutions under certain conditions.

5. The theory is advanced that the fungus secretes an oxidase which acts upon lignin and pentosans to produce acid. Further simultaneous action of oxidase and acid completes the process of decomposition in which the cellulose proper is depleted.

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CXXXVIII. THE DISTURBANCE OF METABOLISM PRODUCED BY BONY AND NON-BONY INJURY, WITH NOTES ON CERTAIN ABNORMAL CONDITIONS OF BONE.

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(*Received May 18th, 1930.*)

The present communication is an attempt to elucidate certain of the grosser changes in the mineral metabolism of the body during the healing of a fractured bone, and to describe certain of the phenomena associated with tissue injury.

Practically all previous investigations have centred round the relative and absolute amounts of calcium and phosphorus in the blood during different phases of the healing process. The results of the various investigators in this field have been very contradictory. Tisdall and Harris [1922] state that, during the second to sixth weeks of the healing of fractures in adults, the phosphorus content of the serum is raised to about that of childhood and that there is a close parallelism between the concentration of inorganic phosphorus and the formation of new bone. This increased concentration of phosphorus begins to decline towards the normal level about the seventh week. The observations of these two workers on the question of the deviations during non-union were not decisive. Eddy and Heft [1923] confirmed this rise in phosphorus in 23 fracture cases, but found a similar rise in 20 cases involving surgical operations and the healing of tissues other than bone. Moorhead *et al.* [1925] examined a series of 30 fractures. In less than 50 % there was a rise to 5 mg. per 100 cc. serum. Union in some cases was observed with practically no increase of the phosphorus content and non-union in other cases with a high figure. Several cases showed slight variations in the serum-calcium paralleling the phosphorus. György and Sulger [1925] found that the normal phosphorus content of 3 mg. per 100 cc. rises to 5·9 mg. during the healing of a fracture, returning to normal after consolidation. When a fracture failed to unite they found that the phosphorus content had not risen. They also found an increase of phosphorus after osteotomies but not after operations on the soft parts. Satanowski [1925] found in dogs a rise of about 1 mg. 3 days after a fracture. The calcium content remained rather irregular, but slightly below normal. Henderson, Noble and Sandiford [1926] examined 20 cases of un-united fracture where the inorganic phosphorus tended to be about the normal adult content. In two cases only

did it rise to the infancy level. The blood-calcium remained normal. Petersen [1924] studied the calcium and phosphorus of the serum in 9 cases of normally healing fractures, and in 9 un-united cases. This investigator states that the union of a fracture does not occur if the product of Ca × P be less than 30. For active healing he considers that the product must lie between 35 and 40. Seven of his un-united cases went on to solid bony union on administering the defective element. They required about 3 weeks' treatment before the blood-serum showed an increase in the low salt. As a control, a similar number of normal fractures was studied: during the process of union there was little or no increase in the serum-calcium or -phosphorus. On the other hand, Ravdin and Jonas [1926] found that 5 un-united fractures gave a Ca × P product of 30–46, and 10 un-united cases gave products varying from 32–59. The blood estimations were performed 6–12 weeks after the injury.

Reviewing the work on blood analyses, the following points appear to emerge from this rather contradictory mass of evidence.

(1) That there probably is a rise in the plasma- or serum-phosphorus following a fracture, and that later this declines towards the normal level.

(2) That such a rise is not peculiar to fracture cases, but may occur after injury to parts other than bone.

(3) That the serum-calcium remains practically constant during the healing process.

As it appeared likely that further investigations along similar lines would yield little evidence of value, it was decided to undertake a series of metabolic examinations of the mineral exchange in cases of normally healing fractures, in fractures treated with irradiated ergosterol, in un-united fractures, in fractures complicated by bony abnormalities, and in wounds of non-bony parts.

General plan of the experiments.

In the majority of the fracture cases the metabolic data were collected during two periods. As it was generally the third day following the accident before the patients were admitted to the experimental ward, and as 3–4 days were then allowed on constant diet before the collection of excreta was commenced, the earlier metabolic periods date from the sixth or seventh day following the accident. The period of collection was 6–7 days. 5–9 weeks later a similar period was studied.

The daily excretion of a few cases of fracture and non-bony injury was examined as far as possible from the date of the accident. All the fractures were simple with the exception of case 15.

In addition to the fracture cases several other pathological conditions of bone have been examined and the metabolic data are described in so far as they throw light on certain of the phases of calcium metabolism.

Determinations of the inorganic phosphorus, non-protein-nitrogen and urea of the blood were made on a few cases of fracture and on a few cases of non-bony injury.

The daily intake of food was selected from the following list:

Dried milk (made up in fluid form from "Cow and Gate" half-cream dried milk).
Brown bread.
Digestive biscuits.
Fresh butter.
Steak (served as stew).
Dried apples (served stewed).
Dried tomato soup (served as soup).
Dried egg (served as custard).
Tea.
Sugar.
Marmalade.
Orange juice.

Varying amounts of all these items were consumed.

With the exception of cases 18, 21, 32 and 34 the subjects were males.

Analytical methods.

The subjects of these experiments were generally in good health apart from their tissue injury. They were allowed to select the quality and quantity of foodstuffs required from a prepared list. This intake, as well as the intake of water, was kept as constant as possible. The urine was collected in 24-hourly specimens, made up to a constant volume and preserved with thymol and chloroform. The various periods during which faeces were collected were marked off with carmine, taken in the form of capsules. The faeces were dried on a steam-bath and in an air-oven before being stored in sealed bottles. The intake of mineral matter was determined at first by summation of the individual food values, but later an average half-day's intake was dried and analysed.

(*a*) Urine:

Total nitrogen	Kjeldahl method
Urea	Urease method
Ammonia and creatinine	Folin's methods
Total sulphur	Denis's modification of Benedict's method
Total phosphorus	Neumann's wet ash method
Calcium	Shohl and Pedley's method [1922]

(*b*) Faeces and food:

Total nitrogen	Kjeldahl method
Phosphorus	Material ashed in the presence of excess $Ca(OH)_2$: phosphorus precipitated as ammonium phosphomolybdate and weighed directly

(*b*) Faeces and food (*contd.*):

Calcium	Material ashed, any iron removed, then Shohl and Pedley's method applied
Sulphur	Preliminary oxidation with nitric acid and bromine. Then Denis's modification of Benedict's method applied

(*c*) Blood:

Non-protein-nitrogen and urea	Folin and Wu methods
Inorganic phosphorus	Recent modifications of the Bell and Doisy technique

Analytical data.

The actual data only will be described here, all discussion being delayed till later. It is necessary to bear in mind that, whereas the bony skeleton contains approximately 97–98 % of the total calcium of the body, it only contains two-thirds of the total phosphorus; the remaining one-third is to be found in the muscles, glandular organs, etc.

A. *Normally healing group of fractures* (1–7) (*vide* Tables I and II).

Calcium. The urinary excretion of CaO varies from 0·0066 g. per kg. per day (p.k.p.d.) to 0·0128 g., with a mean value of 0·0092 g. during the second week of healing; and from 0·0057 g. to 0·0103 g. with a mean value of 0·0087 g. during the fifth to ninth weeks. There is a degree of constancy in this urinary excretion and such variability as exists does not appear to bear any relationship to the intake as defined by the range 0·029 g. to 0·048 g. p.k.p.d., and by a similar range during the second period.

Givens [1918] found that in healthy adults on diets containing more calcium than magnesium, the urinary output of CaO varied from 0·17 g. to 0·66 g. per day. Nelson [1920] stated that in adult men the urinary excretion of CaO varies from 0·12 g. to 0·58 g. per day when the diet is unrestricted. Unfortunately the weights of the subjects of these experiments are not recorded. On the assumption that the average weight would be about 70 kg., the maximum excretion of CaO would be approximately 0·009 g. p.k.p.d., a figure corresponding to the average value of the fracture group. Data are presented here of a few normal subjects (group G) selected from various experimental groups. It is unfortunate that the daily intakes 0·017 to 0·029 g. p.k.p.d. are considerably less than in the fracture group. The urinary excretion of CaO varied from 0·0019 to 0·0057 g. p.k.p.d. with an average of 0·0043 g. These values are distinctly lower than for the fracture group.

The faecal excretion of CaO remained fairly constant during the healing process, being slightly greater during the later periods. On balancing intake with output in this normally healing group, it is obvious that the data reveal positive and negative retentions both in the earlier and later periods. On the average these fluctuations tend to compensate.

Table I. *Daily mineral exchange (g.).*

Case	Period	Wt.	Age	Fracture	Time in days	Intake of N	Output of N		Balance of N	Intake of CaO	Output of CaO		Balance of CaO	Intake of P_2O_5	Output of P_2O_5		Balance of P_2O_5
							Urine	Faeces			Urine	Faeces			Urine	Faeces	
1	A	65	21	Tibia	5–10	—	—	—	—	3·13	0·43	—	—	4·69	4·09	—	—
	B				32–33	—	—	—	—	3·10	0·67	2·73	− 0·30	4·67	2·36	3·16	− 0·85
2	A	67	40	Femur	11–17	—	—	0·99	—	2·03	0·52	1·51	0·00	2·69	2·90	1·67	− 1·88
	B				42–47	—	—	1·15	—	2·06	0·59	1·68	− 0·21	3·16	2·06	1·39	− 0·29
3	A	60	32	Femur	6–13	10·63	15·77	2·20	− 7·34	2·12	0·77	1·79	− 0·44	3·47	3·87	1·87	− 2·27
	B				36–41	10·60	8·97	1·63	0·00	2·11	0·61	1·71	− 0·21	3·47	2·57	1·19	− 0·29
4	A	71	40	Femur	5–12	9·49	14·75	0·83	− 6·09	2·08	0·75	1·29	+ 0·04	3·20	4·09	1·43	− 2·32
	B				40–45	9·49	9·52	1·27	− 1·30	2·08	0·65	1·46	− 0·03	3·20	2·04	1·31	− 0·15
5		67	34	T. & F.	4–10	—	—	—	—	3·25	0·47	2·69	+ 0·09	4·59	3·46	2·59	− 1·46
6		71	62	Femur	56–63	13·28	12·74	0·43	+ 0·11	2·19	0·57	1·34	+ 0·28	3·86	2·505	0·96	+ 0·39
7	A	60	19	Femur	6–12	—	—	—	—	2·05	0·47	1·06	+ 0·52	2·61	3·60	1·06	− 2·05
	B			Radius	34–40	—	—	—	—	2·05	0·34	1·35	+ 0·36	3·16	2·33	1·28	− 0·45
8		65	23	Femur & patella	8–16	—	—	—	—	2·11	0·48	1·71	− 0·08	2·97	3·72	1·86	− 2·61
9		40	19	Femur	7–14	—	—	—	—	2·96	0·74	2·23	− 0·01	4·46	3·61	2·30	− 1·45
10		65	31	T. & F.	6–13	—	—	—	—	1·39	0·65	0·74	0·00	2·29	3·10	0·62	− 1·43
11	A	69	29		13–20	—	—	—	—	3·13	0·91	2·67	− 0·45	5·26	3·88	2·84	− 1·46
	B				34–39	—	—	—	—	3·18	1·02	2·85	− 0·69	5·43	3·55	2·90	− 1·02
12		62	48	T. & F.	7–14	—	—	—	—	2·01	0·22	1·31	+ 0·48	2·71	2·30	1·10	− 0·69
13		64	23	T. & F.	70–77	—	—	—	—	3·25	0·49	2·36	+ 0·40	4·59	2·60	2·06	− 0·07
14		54	16	Femur	28–35	—	—	—	—	2·00	0·55	1·28	+ 0·17	2·86	1·87	1·09	− 0·10
15		60	23	T. & F.	196–202	9·55	9·70	1·28	− 1·43	2·09	0·66	1·79	− 0·36	3·25	2·75	1·03	− 0·53
16	A	60	46	T. & F. & humerus	70–77	8·23	7·05	0·53	+ 0·65	2·05	0·51	1·26	+ 0·28	2·97	1·95	0·78	+ 0·24
	B				105–112	—	—	—	—	2·07	0·49	1·12	+ 0·46	3·05	1·76	0·87	+ 0·42
17		64	33	Femur	42–49	—	—	—	—	2·15	0·70	1·485	− 0·035	3·75	2·295	0·925	+ 0·53
19		68	40		6–11	10·33	12·60	1·52	− 3·79	2·19	0·50	2·07	− 0·38	3·47	2·79	1·86	− 1·18
20		60	18		5–12	9·98	13·65	1·75	− 5·42	2·10	0·43	1·70	− 0·03	3·26	2·77	1·69	− 1·20
21		41	14		5–12	8·75	9·45	—	—	2·05	0·43	—	—	—	—	—	—
22		55	21		3–18	—	—	—	—	1·97	0·38	—	—	4·88	3·28	—	—
23		67	40		—	13·29	12·64	1·28	− 0·63	1·92	0·37	1·65	− 0·10	3·76	2·66	1·18	− 0·08
24		68	19		—	13·51	13·23	1·16	− 0·88	1·21	0·39	0·81	+ 0·01	—	—	—	—
25		68	40		—	13·28	12·86	1·17	− 0·75	1·96	0·385	1·54	+ 0·035	3·79	2·53	1·30	− 0·04
26		71	62		—	—	—	—	—	1·54	0·14	—	—	4·26	2·81	—	—
27		60	25		—	—	—	—	—	1·47	0·25	—	—	4·44	3·00	—	—
28		70	40		—	—	—	—	—	1·18	0·22	0·91	+ 0·05	—	—	—	—

"A" refers to early period. "B" refers to late period. Weights are expressed in kg. "Time in days" expresses time in days from date of accident and duration of the period during which excreta were collected. "T. & F." = tibia and fibula.

Table II. *Average daily mineral exchange of CaO and P_2O_5 in the various groups expressed as g. per kg. body-weight.*

Group	Period	Intake CaO	Output CaO		Balance CaO	Intake P_2O_5	Output P_2O_5		Balance P_2O_5
			Urine	Faeces			Urine	Faeces	
A	Early	0·0355	0·0092	0·0257	+ 0·0006	0·0510	0·0554	0·0265	− 0·0309
	Late	0·0345	0·0087	0·0263	− 0·0005	0·0548	0·0354	0·0237	− 0·0043
B	Early	0·0375	0·0108	0·0287	− 0·0020	0·0584	0·0557	0·0297	− 0·0270
	Late*	0·0475	0·0152	0·0425	− 0·0102	0·0810	0·0529	0·0433	− 0·0152
E	Early	0·0384	0·0079	—	—	0·0646	0·0491	—	—
G		0·0230	0·0043	—	—	0·0613	0·0418	—	—

* Only one case.

Phosphorus. It is evident from Tables I and II that there is some gross disturbance of phosphorus metabolism, particularly during the earlier stages of the healing process. The average daily intake of P_2O_5 per kg. during the second week was 0·051 g. and during the second metabolic period 5–7 months later the intake was only slightly greater, 0·0548 g. During the earlier period the average urinary output of 0·0554 g. p.k.p.d. was greater even than the intake. During the later period the urinary output had declined to 0·0354 g. p.k.p.d. The loss to the body during these two periods was 0·0309 g. and 0·0043 g. p.k.p.d., that is, the loss was approximately seven times greater during the earlier period. The faecal excretion of P_2O_5 is also greater during the earlier period. The main excretory route for this excessive phosphorus loss is, however, the kidneys.

Nitrogen and sulphur. When the regular occurrence of this phosphorus loss was noted, the nitrogen balance was determined in a few of the remaining cases. In case 3 there was an average daily loss of 7·34 g. N during the second week. 5–6 weeks later the output balanced the intake. The P_2O_5 loss per day during the same periods was 2·27 g. and 0·29 g. respectively. The daily loss of N in case 4 was 6·09 g. during the second week and 1·30 g. during the seventh to eighth weeks. The P_2O_5 loss during these two periods was 2·32 and 0·14 g. respectively. Case 6 showed positive retentions of 0·11 g. N and 0·39 g. P_2O_5 per day during the eighth week. This subject had been massaged for a week previously.

Table III. *Case 29. The daily urinary excretion of an adult man with a fractured femur, dating from the second day following the accident.*

Daily excretion in g.

Days	Urine		Days	Urine	
	Total N	Total P_2O_5		Total N	Total P_2O_5
2	7·92	1·91	8	19·9	3·65
3	24·9	3·10	9	16·7	3·16
4	32·5	3·88	10	16·7	3·16
5	25·2	4·75	11	14·0	2·73
6	22·7	4·75	12	15·1	2·78
7	21·0	3·33			

Daily intake of N = 14 g.

Table IV. *Case 30. This subject, a boy aged 15 years, was subjected to a double osteotomy of his femor for the correction of a deformity, on the third day of diet.*

Daily excretion in g.

	Urine					
Days	Total N	Urea-N	Ammonia-N	Total SO_3	Inorganic SO_3	Total P_2O_5
1	10·08	7·96	0·437	1·965	—	2·46
2	10·08	8·14	0·430	1·86	—	2·60
3	6·72	4·93	0·277	1·39	—	2·26
4	13·96	11·53	0·781	2·50	1·77	2·88
5	12·39	10·06	0·437	1·99	1·69	2·54
6	11·76	9·35	0·596	2·00	—	2·68
7	12·73	8·60	0·437	1·935	—	2·07
8	10·30	8·26	0·555	2·08	—	2·10
9	12·39	9·01	0·403	2·15	—	2·245
10	12·49	—	—	—	—	—
11	12·18	—	—	—	—	—
12	11·23	—	—	—	—	—
13	11·44	—	—	—	—	—
14	11·13	—	—	—	—	—
15	11·55	—	—	—	—	—
16	11·23	—	—	—	—	—

Daily intake of N = 11·78 g. (except third day when diet was reduced).
This was the day of operation.

Table V. *Case 31. The daily urinary excretion of an adult man from the third day following a spiral fracture of the tibia caused by a fall of 3 ft. only.*

Daily excretion in g.

	Urine		
Days	Total N	Total SO_3	Total P_2O_5
3	12·2	2·07	2·41
4	14·1	2·57	3·09
5	14·5	2·79	3·24
6	17·4	2·83	3·64
7	16·1	3·10	3·46

Daily intake of N = 9·12 g.

Table VI. *Case 32. The daily urinary excretion of an adult woman from the second day following a fracture of the tibia.*

Daily excretion in g.

Days	Urine Total N	Days	Urine Total N
2	9·24	7	13·38
3	11·865	8	9·03
4	10·99	9	9·87
5	12·18	10	9·36
6	13·28		

Daily intake N = 10·72 g.

Tables III, IV, V, VI and VII indicate the day-to-day changes in the urinary constituents following the fracture of bone. While the majority of the fractures were manipulated and set with the patient under the influence of an anaesthetic, case 33 who presented the greatest catabolism of the series had no such treatment.

Table VII. *Case* 33. *The daily excretion of a youth aged* 16 *years, from the third day following a fracture of both bones of one leg.*

Daily excretion in g.

Days	Vol. of urine (cc.)	Total N Urine	Total N Faeces	Total SO_3 Urine	Total SO_3 Faeces	Total P_2O_5 Urine	Total P_2O_5 Faeces	S : N ratio of excess output		P_2O_5 : N ratio of excess output	
3	630	12·02		2·61		3·48					
4	990	23·24		3·76		4·36		1 : 14·44		1 : 5·96	
5	1480	32·06		4·96		5·38					
6	1230	25·20		3·81		4·08					
7	1690	27·58		4·36		4·71					
8	1680	24·01	Daily	3·98	Daily	4·11	Daily	1 : 13·70	1 : 13·09	1 : 6·43	1 : 6·19
9	1390	19·25	av.	3·43	av.	3·78	av.				
10	1100	15·54	1·514	2·74	0·385	3·115	1·406				
11	1580	19·95		4·68		4·41					
12	1380	20·93		3·64		3·60		1 : 11·01		1 : 6·24	
13	1140	17·78		3·00		3·215					
14	1030	18·34		3·09		3·20					
Intake		11·70		1·931		3·560					

The S : N and P_2O_5 : N ratios of the excess outputs were determined in case 33. The figures in Table VII are the ratios of the loss taken in 4-day periods and also the total loss to the body during the period of observation—12 days.

B. *Group of fracture cases treated with irradiated ergosterol* (8–11).

Calcium. There was a tendency in this group for an increased urinary excretion of calcium to occur both in the earlier and later stages of repair. The mean urinary output of CaO during the second week was 0·0108 g. p.k.p.d., the intake being 0·0375 g. p.k.p.d. There was also a more general tendency for a negative retention to occur in this group. The active substance was administered in the form of "Radiostol" pellets (British Drug Houses, Ltd.). Each pellet is said to contain 6000 antirachitic rat units. Three of the cases received two pellets daily; case 10 received four.

Phosphorus. It was found in this group also that there was a distinct loss of phosphorus, particularly during the earlier period, the average daily loss being 0·027 g. p.k.p.d. Again the main excretory route for this catabolite was the kidneys.

C. *Group of un-united fracture cases* (12–15).

An attempt was made to determine if there were any gross deviations of the mineral metabolism in a group of un-united fractures. This group did not include such cases as are termed "pathological fracture," but consisted of a group which for unknown reasons had failed to heal.

The mineral metabolism of case 12 was examined during the second week following the re-fracture of a 9 months-old mal-united fracture. The patient had been in bed for a fortnight prior to the operation, and prior to that had moved about on crutches. The noticeable feature in this case was the absence of the marked phosphorus loss. Case 13 was a 10 weeks-old fracture of both

bones of the leg and case 15 was a compound fracture of both bones of the leg of 7 months' standing. This case showed slight negative retentions of calcium and phosphorus, and there was gross rarefaction of the bones visible by X-ray. At the operation performed 3 weeks later the friable nature of the bones was quite obvious. Case 14, a girl aged 16 years, fractured the neck of her femur by the simple process of knocking her toe against the pavement kerb. After 3 months there was still some degree of non-union, but eventually the bone healed. The X-ray evidence did not point to any bony abnormality.

D. *Group of pathological fractures* (16–18).

This group consists of three members. The first case (16) was a man who exhibited the triad of blue sclerotics, otosclerosis and bone fragility. He had suffered from four previous fractures, all of which had healed normally. His last injury (fifth) involved both bones of one leg. The tibia was fractured in two places, other cracks also being visible. The fibula was broken in one place. In addition, there was an impacted fracture of the surgical neck of the left humerus. These injuries were the result of a severe accident. Eleven weeks after the accident, although the humerus had appeared to unite well, there was still no union of the leg bones and no X-ray evidence of callus formation. Following the first experimental period, irradiated ergosterol was administered (three "Radiostol" pellets daily). A few weeks later definite callus formation was visible. A second metabolic period was instituted 1 month after the first. The urinary excretions in both periods were of the same order as were found in the normal fracture group. There was a small but definite retention of both calcium and phosphorus during the first period and this was increased considerably during the second, the CaO retention rising from 0·0047 g. to 0·0079 g. and the P_2O_5 from 0·0040 g. to 0·0070 g. p.k.p.d. Some degree of union took place, but the recovery was imperfect. The capacity for bone healing in osteogenesis imperfecta is supposed to be unimpaired and the unsatisfactory result in the above case is probably to be attributed to the badly fractured bone and not to any intrinsic cause.

The abnormality of the second case (17) was only discovered accidentally on X-raying the patient. The patient appeared to be in perfect health, but the X-ray plate showed marked fibro-cystic changes in the right femur and left ileum. There were slight changes also visible in the left femur and pelvis generally. No other evidence of bony changes was present. The Wassermann reaction was negative. The right femur which was fractured healed perfectly, the injured leg having been massaged from the third day onwards. The mineral metabolism showed no deviation from that of the normal fracture group. There was, however, a small retention of phosphorus. A similar retention was found in case 6 of the normal group. Both of these cases received massage. Work on the influence of massage is being delayed for a subsequent communication.

The third case (18) (Table VIII) of this series also showed fibro-cystic changes in the bones, particularly the pelvis. The cyst-like areas were numerous,

small and ill-defined in the pelvis, but more obvious in the femora. This patient had passed successfully through five pregnancies, but while out walking 7 weeks after the birth of the sixth child she felt a sudden pain over the symphysis pubis and had to be carried home. Double fractures of the rami of the os pubis and ischium were found. After $4\frac{1}{2}$ months in bed she was able to walk about 20 yards, her gait being more a waddle than a walk. This case had a defective dietary history. She did not eat butter, and milk was taken only in her tea. Whilst pregnant she usually took buttermilk and some butter. Metabolic periods were held 5, 7 and 19 months after the original fracture (*vide* Table VIII). Following on the first metabolic period, irradiated ergosterol was administered daily (four pellets of "Radiostol"). While there was initially a slight calcium retention and phosphorus loss (the patient having been in bed for some time previously), the administration of the active substance caused marked retentions of calcium and phosphorus which were maintained for over a year. At first the retention of phosphorus was relatively greater than that of calcium, but this was due, in part, to the fact that the patient had recovered sufficiently to be able to walk about more freely. The disused muscles were then able to recover their normal condition. This case ultimately made a very satisfactory recovery although she still has occasionally deep-seated pain. (A fuller clinical account of this case will be published elsewhere.)

E. *Group of cases with injury to non-bony parts.*

This group includes a case operated on for the removal of a piece of torn cartilage (19), a case of muscle laceration (20), a case of muscle incision (21), and a perforating spike wound of the leg (22).

Calcium. The urinary excretion of calcium is not much lower than that of the normal fracture group. There is also a tendency for a negative retention to occur.

Phosphorus. There is a marked loss of phosphorus. This loss is not quite so marked as that found to occur during the earlier days of the healing of a fracture. The kidneys again form the chief excretory route.

Nitrogen and sulphur. The daily average loss of nitrogen from the sixth till the eleventh days in case 19 was 3·79 g. per day, the P_2O_5 loss being 1·18 g. The average daily loss of nitrogen in case 20, from the fifth till the twelfth day, was 5·42 g., the P_2O_5 loss being 1·21 g. The P_2O_5 : N ratios of these excess outputs are 1 : 3·2 and 1 : 4·7 respectively.

The daily urinary excretion of sulphur (SO_3), nitrogen and phosphorus (P_2O_5) of case 22 is represented in Table IX. This is a record from the second day following the accident.

F. *Group of cases presenting abnormal mineral metabolism.*

Brief mention only will be made of these cases and only in so far as they concern the present work.

It is hoped to publish fuller details of case 34 in the near future. This case when first seen at the age of 18·5 years had been unable to walk for 5 years

Table VIII. *Daily mineral exchange (g.).*

Case	Period of collection	Intake of CaO	Output of CaO		Balance of CaO	Intake of P_2O_5	Output of P_2O_5		Balance of P_2O_5
			Urine	Faeces			Urine	Faeces	
18	1. Ordinary diet	1·82	0·38	1·36	+ 0·08	2·75	1·66	1·17	− 0·08
	2. Ordinary diet + irradiated ergosterol (2 months' treatment)	1·95	0·29	1·15	+ 0·51	—	—	—	—
	3. Ordinary diet + irradiated ergosterol (17 months' treatment)	1·72	0·35	0·71	+ 0·66	2·48	1·34	0·57	+ 0·57
34	1. Ordinary diet	1·01	0·18	0·76	+ 0·067	1·61	0·64	0·97	0·00
	2. Ordinary diet + calcium glycerophosphate	1·005 + 1·02	0·16	1·58	+ 0·285	1·61 + 1·29	—	1·08	—
	3. Ordinary diet + calcium glycerophosphate + irradiated ergosterol	1·06 + 1·64	0·30	0·65	+ 1·75	1·77 + 2·08	1·48	0·57	+ 1·80
	4. Ordinary diet + phytin + irradiated ergosterol	1·06 + 1·12	0·26	0·89	+ 1·03	1·77 + 3·36	1·71	1·74	+ 1·68
35	Ordinary diet	2·845	0·166	2·26	+ 0·42	4·35	1·79	2·35	+ 0·21

Table IX. *Case* 22.

Days	Daily excretion in g. Urine: Vol.	Total N	Urea-N	Creatinine-N	Total SO_3	Total P_2O_5
2	960	17·85	14·70	0·79	2·88	2·63
3	880	18·27	15·39	0·84	3·18	0·17
4	1130	16·90	14·45	0·84	3·00	4·05
5	1090	14·49	—	0·615	2·64	4·10
6	1010	14·80	—	0·52	2·53	3·26
7	1080	13·44	—	0·58	2·30	3·08
8	1500	12·39	—	0·50	2·23	3·07
9	1270	11·71	—	0·72	2·03	2·86
10	—	11·55	—	0·63	2·09	2·97
11	1100	11·33	—	0·72	2·20	3·10
12	1150	10·29	—	0·58	2·08	2·47
13	1388	10·60	—	0·615	1·90	2·41
14	1490	11·34	—	0·56	1·94	2·84
15	1330	10·71	—	0·63	—	3·16

Daily intake of N = 14·35 g.

owing to weakness and pain of her thighs and legs. She had little, if any, interest in her food, and her intake of minerals had been particularly low. It was difficult to arrive at a diagnosis of her condition. The X-ray plates showed changes somewhat similar to those found in late rickets and osteomalacia but yet they were not typical. The administration of irradiated ergosterol (3–4 pellets "Radiostol" daily) caused very marked clinical improvement and by the end of $2\frac{1}{2}$ months the patient was able to walk with considerable freedom. The inorganic phosphorus of the whole blood remained persistently low

(1·4–1·6 mg. per 100 cc.), but the calcium of the serum did not fall below 9·35 mg. per 100 cc. at any time.

A clinical description of the next case (35), one of osteitis deformans, has already been published [Cuthbertson, 1927] and the analytical data presented here are abstracted from that paper.

G. *Control group of healthy adults.*

Several of these cases had been in bed for varying periods with one leg fixed in a splint. The metabolism of case 23 was examined during a period of 2 days' activity followed by a week's rest in bed with one leg in a splint. Case 24 was examined during a period of 9 days' confinement in a splint, and case 25 during a period of 12 days' similar confinement. The data presented are the average daily metabolic exchanges during the particular period. These figures are abstracted from a previous communication on the effect of disuse on metabolism. Cases 26–28 were not confined to bed and led active lives.

Discussion.

Before attempting to discuss the observed phenomena, it is first of all necessary to recall the chief events in the healing process. Without reference to any particular theory, it is held that about 4–6 days after an injury involving the fracture of bone, there are found areas of denser staining in the semi-elastic blood clot, which apparently represent the beginning of the deposition of calcium salts. In about 10 days, the early callus, a thick tenacious material, joins the bone ends and invades the adjacent muscle bundles. After about a month this callus has been absorbed or altered and there is in its place a firm mass which allows of only slight movement. Later this ill-defined callus assumes more the natural shape and consistency of the bones and union becomes more complete. Marked changes occur at the ends of the fractured bones. There is an apparent absorption of both matrix and cells—a process of demineralisation and atrophy. Leriche and Poligard [1928] believe that the genesis of new bone trabeculae is conditioned by the absorption of the pre-existing bone, and that this process of rarefaction begins immediately after the trauma. There is thus a process of bone absorption coincident with an anabolic phase. Where apposition is poor the bones may waste themselves on an impossible task. In case 11 the apposition appeared to be fairly good, and there was a definite formation of callus visible on the X-ray plate; yet the bones had not united, although there was no apparent cause. Gross rarefaction, particularly of the proximal fragment of the femur, was clearly visible and union ultimately took place after the two fragments were plated. Such a wasting was also visible in the X-ray plates of case 15. At the operation performed 7 months after the injury there was no cause for non-union, other than the separation of the fragments. Very little callus had been laid down and the bones were soft. The bone ends were freshened by shaving the apposed surfaces, and the bones plated. The bone was so soft that the nails held their position with great difficulty.

Union followed. A fragment from the end of the fibula was analysed and it was found to have a CaO : P_2O_5 ratio of 1·43 : 1. The normal ratio for bone is 1·47 : 1 assuming the percentage of CaO and P_2O_5 to be 28·85 % and 19·55 % respectively [McCrudden, 1910]. A piece of callus removed at open operation on a case of badly apposed bone fragments showed a ratio of 2·3 : 1. This would appear to support the view put forward by Schwarz, Eden and Herrmann [1924] that in ossification calcium is first laid down in organic combination, and that subsequently union with phosphorus occurs.

From the available data presented in this report the following observations can be made.

Calcium. The metabolism of calcium as determined by the intake and output of CaO during the different stages of the healing process in fracture cases shows but little deviation from the normal. Although calcium may sometimes be retained in small amount there is as often a tendency to a slight but continuous loss. It is difficult to trace any relationship between the positive and negative retentions and the phase of healing.

It has long been known that certain substances, *e.g.* milk, various calcium salts, etc., can increase the urinary output of CaO. Table X illustrates the effect of the administration of 10 g. calcium glycerophosphate on two consecutive days to a patient with a fractured spine, who had been in bed for a month previously. The increased excretion was however slight. Curiously enough, there was a fall in the urinary excretion of phosphorus.

Table X. *Case* 35. *Urine.*

		Urine		
Days	Intake CaO (g.)	Output CaO (g.)	Intake P_2O_5 (g.)	Output P_2O_5 (g.)
1	1·065	0·476	4·54	3·31
2	1·065	0·483	4·54	3·18
3	1·065	0·476	4·54	3·57
4	1·065	0·497	4·54	3·32
5	1·065	0·465	4·54	3·35
6	1·065	0·511	4·54	3·50
7	1·065	0·476	4·54	3·50
8	1·065 + 1·224	0·546	4·54 + 1·556	2·73
9	1·065 + 1·224	0·586	4·54 + 1·556	3·07
10	1·065	0·553	4·54	3·82
11	1·065	0·533	4·54	3·70
12	1·065	0·513	4·54	3·62

While it has been suggested that the kidney supplies the sole, or at any rate the main, route for the excretion of calcium [Telfer, 1922], the recent work of Grosser [1920], Husband, Godden and Richards [1923], Bergeim [1926], and Stewart and Percival [1927] has indicated that the main excretory route is probably through the wall of the large intestine, and that the urinary calcium represents but a small fraction of the total excretion.

The urinary excretion of CaO cannot be taken as an indicator of the phase of calcium metabolism, for an increase may be found when bone is rarefying and when it is being laid down. In osteomalacia, it is said that the amount of calcium passed in the urine may be so great that it is visible to the naked eye as renal

sand. In case 34 there was an increased urinary excretion only when there was a coincident increased retention. Mere increasing of the intake of lime was ineffective in this case without the addition of irradiated ergosterol. Further, a decrease of the urinary excretion of calcium may be found either when bone is being laid down in excess as in certain phases of osteitis deformans (case 35), or in rickets where there is a primary or secondary diminished absorption.

The average urinary calcium excretion of the fracture cases treated with irradiated ergosterol was 0·0108 g. p.k.p.d. This increase which results from the administration of the irradiated substance is not much greater than the value found in the untreated group. The ergosterol may merely accelerate the normal process. Harris [1930] quotes the unpublished observations of Watchorn to the effect that this irradiated substance causes a marked increase in the urinary excretion of calcium.

This increased urinary calcium excretion in the fracture cases is not coincident with an increased retention of calcium or phosphorus, for there is often a slight loss. This finding coupled with the effect of irradiated ergosterol on the metabolism of case 34 would appear to furnish some evidence in support of the theory that the primary effect of this vitamin is on the absorption of mineral matter from the gut, rather than a direct stimulation of the tissues to calcify. This increased absorption may result irrespective of any increased demand from the skeleton. On the other hand, it may be that this active agent has some effect on the intermediary metabolism, as has been suggested by Hess [1929].

In a fracture the whole healing process may be purely local at first, the mineral elements coming from the adjacent bone fragments, and only subsequently is there an actual retention of minerals to make good the depleted bone. There is probably in addition a slight loss due to disuse of the body generally which may be masked by an increased deposition at the site of fracture. Another possible explanation would demand that in addition to an active demineralisation resulting in a loss of mineral salts, there is active deposition in the junctional area, the two processes not however being in the nature of a single reversible reaction, but separate, the deposited substance not necessarily coming from the atrophied bone. On the available evidence it is impossible to decide which is the natural method. The latter would be a distinct disadvantage to a wounded animal cut off from its food supplies, and would not be in the interests of natural economy.

Phosphorus, nitrogen and sulphur.

While the metabolic exchange of calcium shows only a slight deviation from the normal, that of phosphorus is very definitely altered. There is an excessive loss of this element during the earlier phases of the healing process. By the end of 1–2 months there is only a slight loss. The main excretory route for this excess output is by the kidneys. During the second week of healing there is, on the average, seven times as much phosphorus being lost as during the fifth to eighth weeks.

It is obvious that bone cannot be the main source of this phosphorus, for the loss is too early and too great, and further it is coupled with an excessive loss of nitrogen and sulphur. The main source of these catabolites cannot be directly from the absorption and the elimination of the effused blood at the site of fracture, for the loss is too great.

There remains for consideration the muscular system. The question naturally arises as to what disturbance of muscular metabolism would lead to such an enormous and early loss of substance. Tables III and VII illustrate the rates of excretion of nitrogen, phosphorus and sulphur following the fracture of bone. These cases presented the greatest catabolism of the series. They demonstrate how early this loss reaches its maximum. On the other hand Tables IV, V and VI present excretory rates which are not of a much greater magnitude than are found in cases of pure disuse due to immobilisation in splints. The S : N and P_2O_5 : N ratios of the total excess output in case 33 were 1 : 13·09 and 1 : 6·19 respectively during the period of observation. It will be noted that during the later days there is a relatively greater output of sulphur in relation to the loss of nitrogen. The phosphorus output, on the other hand, maintained a more constant relationship to the nitrogen. The generally accepted S : N and P_2O_5 : N ratios for muscle are 1 : 14 and 1 : 6·6 respectively. Although due consideration must be allowed for the shortness of the period of observation the ratios found in case 33 suggest that the main source of the substance catabolised is some sulphur-rich complex store material, which is probably intimately connected with muscle.

It has long been observed that disuse of muscle leads to atrophy, and the natural question arises as to the rôle played by pure disuse in the disturbed metabolism of fracture cases. This question has, in part, been already investigated by the present writer [Cuthbertson, 1929], who found that normal subjects in nitrogenous equilibrium showed within a day or two from the commencement of a period of muscle rest involving fixation of a lower limb in a splint coupled with rest in bed, a rise in the excretion of sulphur, nitrogen, phosphorus and later calcium in that order of priority. This rise was maintained fairly steadily for a varying period after which it gradually declined. The S : N ratio suggested a sulphur-rich source for the excreted material which was presumably muscle. While the loss of sulphur, nitrogen, and phosphorus during such an experimental period of imposed rest, though considerable, does not reach such a high value as is found following a fracture of bone there appears to be some qualitative resemblance. That the degree of immobilisation is considerably more absolute in the case of a fracture and may lead to a much earlier and greater loss of muscle substance is very probable, but this explanation does not appear to be sufficient adequately to account for the loss found to occur in wounds of non-bony parts, where there has been little, if any, fixation. These changes found in cases 19–22 which involve a loss of sulphur, nitrogen and phosphorus were similar to those produced by a fracture, but of less extent. Many years ago Bauer [1872] noted that an increased nitrogen

elimination in the urine is found after blood-letting. The experiments of Turner, Marshall and Lamson [1915] and Taylor and Lewis [1915] illustrate the rapidity of the renewal of the blood constituents. Their experiments indicate an increased metabolism of protein.

Apart from pure disuse there remain two other possible explanations to be considered. It has long been known that muscular wasting associated with bone or joint disease is much more rapid and extreme than that which occurs with simple disuse of a limb. It is also true that there is a more rapid and extreme wasting in limbs immobilised for some inflammatory or traumatic lesion than in limbs immobilised to correct some deformity. The reason for this lies not so much in the degree of immobilisation, but in some reflex trophic effect. Harding [1925, 1926, 1929] extending the observations of Raymond [1890], Hoffa [1890], and Langley *et al.* [1915, 1917] has shown that such an atrophy, which is mainly reflex in character, depends on the integrity of the afferent paths. This theory demands an exhaustion of the muscle by an excessive number of stimuli. The present writer [Cuthbertson, 1929] found that, following a period of simple disuse in a man whose metabolism was normal, operative removal of a fragment of semi-lunar cartilage under spinal anaesthesia resulted in a few days in a very rapidly increasing loss of substance, which was presumably muscle. This excessive catabolism only commenced to fall after the patient had been out of bed for a day and a half. During this post-operative period the patient's legs were only loosely held between sand-bags. It is difficult to suggest any other explanation than that of a reflex trophic effect to account for this considerable loss of substance, as the increased catabolism was far in excess of that produced in an earlier period of experimental immobilisation. May then this increased catabolism found in fracture cases be mainly reflex in origin also?

One other possible contributory condition remains to be examined, namely, toxic waste. Here our knowledge is extremely vague. When tissue is broken down within the body, there are eliminated a great many cellular products whose final elimination is due to an enzymic dissolution. Just as histamine and allied compounds appear to play such a large part in the production of shock, so some allied compounds formed from the breaking-up of the cellular debris in the fractured or injured area may cause a disturbance of the normal balance of the body cells resulting in an imbalance between anabolism and catabolism. A small febrile rise is occasionally found in cases of injury, but it is seldom pronounced if the fracture be simple, and lasts at the most but a day or two. In cases 29 and 33 where the greatest catabolisms were observed, no rise was noted in case 29 and only a rise of 0·5° F. in case 33.

In very general terms the gross disorder of metabolism in fracture cases may be taken to be the result of two effects—disuse atrophy and tissue injury.

Unfortunately this investigation throws little or no light on some of the unknown causes of non-union.

The exhibition of irradiated ergosterol did not appear to facilitate the

healing process in what was presumed to be a normal group of fractures: indeed the clinical after-history was one of delayed union. The group is admittedly too small on which to base any general clinical deductions, but since its members were selected at random it would suggest that this form of therapy should not be indiscriminately used in normal cases without more experimental evidence. Certain cases of pathological fracture and abnormal bone metabolism appear, however, to benefit very markedly from the addition of this vitamin to their diet.

Table XI. *Blood analyses.*

mg. per 100 cc.

Case	Age	Injury or fracture	Time in days between injury and operation	Inorganic P		Nitrogen	
				Plasma	Whole blood	Non-protein	Urea
30	14	Double osteotomy of femora	4	—	4·5	36	20
				—	4·1	29	17
36	39	Humerus	4	—	2·1	29	17
37	35	Tibia	4	3·2	—	35	13
			11	2·5	—	—	—
38	36	Tibia	4	1·5	—	36	—
			10	1·4	—	—	—
39	42	Femur (compound)	21	3·1	—	—	—
40	54	Femur	5	2·8	—	—	—
			21	2·1	—	39	—
41	50	Femur	28	4·2	—	—	—
42	19	T. & F.	3	3·3	—	—	—
			11	4·5	—	—	—
43	21	T. & F.	3	3·9	—	—	—
			11	5·0	—	—	—
44	33	Femur	20	5·3	—	—	—
			29	4·9	4·2	32	—
45	19	Tibiae	60	3·4	—	—	—
46	20	Laceration of leg	7	3·5	—	—	—
			20	3·3	—	44	—
47	17	T. & F.	8	5·0	4·8	30	—
48	30	T. & F.	24	—	1·5	—	—
49	50	T. & F. (compound)	7	3·8	—	39	—
			14	—	3·5	—	—
50	43	T. & F.	21	3·9	—	—	—
51	23	Tibia	7	3·6	—	39	—
			14	—	3·7	60	—
52	22	T. & F. (compound)	21	4·1	4·2	—	—
			28	—	4·2	34	—
53	66	T. & F.	21	—	3·3	—	—
			28	—	3·6	—	—
54	31	Femur	4	3·2	—	31	—
			14	4·4	—	44	—
55	23	T. & F.	3	3·6	—	—	—
			14	3·8	—	41	—
56	23	Tibia	7	4·0	—	36	—
57	37	Laceration of leg	7	2·4	—	32	—

T. & F. = tibia and fibula.

Blood analyses.

As was suggested earlier in this paper, the work of the various investigators on the inorganic phosphate of the blood in fracture cases indicates that there probably is a rise in the level of this element during the earlier weeks of healing. The evidence also suggested that such a rise was not specific for wounds of bone, but might follow non-bony injury. When it became evident that there was an early and very considerable loss to the body of sulphur, nitrogen and phosphorus, the question naturally arose as to whether the rise in the blood-phosphorus might also be an indicator of the increased catabolism. If such were the case then one might also expect to find an increase in the inorganic sulphur and non-protein nitrogen in the blood. The present series of blood analyses are concerned with this question. They were performed at varying intervals from the date of injury, and with few exceptions all were from adults. The patients had fasted for 12 hours previously.

It is generally held that there is an approximately even distribution of inorganic phosphorus between corpuscles and plasma, the upper limit of normality being 3·5 mg. per 100 cc. The data indicate that in many cases there is a rise in the inorganic phosphorus to over 3·5 mg., but that healing may occur without such a rise. This rise is not contemporaneous with the maximum catabolism noted in the urine, but occurs during the second and third weeks, declining to the normal later. There appears to be no coincident rise in the non-protein-nitrogen or urea of the blood although subsequently there is a tendency for the values to approach the upper limits of normality. Further work is being done to determine the significance of the changes which occur during the later weeks, for they seem to be intimately connected with the process of repair, but whether of bone or soft tissue or more probably both it is at present impossible to say.

Conclusions.

In conclusion it seems reasonable to seek an interpretation for the biological significance of these phenomena associated with injury. Why is there such an early and rapid catabolism of the tissues? Further, is there any significance to be attached to the fact that the evidence of maximum catabolism is contemporaneous with the first signs of regeneration of the injured part?

Injury to an animal generally leads to lessened activity and a lessened activity usually results in a diminished food supply. The necessity is urgent, and the body may require to catabolise its reserves to meet both the exigencies of repair and of maintenance.

In simple disuse the catabolic disturbance, although not of the extent sometimes found associated with injury, appears to be qualitatively the same. Similarity of response, however, must not be taken as an indication that the primary causes are identical. Simple disuse, that is disuse not caused by any

abnormal condition of the tissues, is largely an experimental state, and is seldom found in nature apart from injury. As disuse leads to decreased activity, it is again essential for the animal to economise. The non-functioning part may require to be sacrificed for the whole.

It is possible that some such hypothesis of urgency may account for the relatively increased protein catabolism found to occur during the second to fourth days in the majority of starved subjects [Luciani, 1890; Munk, 1893; Johansson *et al.* 1897; Benedict, 1907, 1915; Cathcart, 1907]. The general assumption is that the protein reserves are being catabolised in an endeavour to maintain a constant level of metabolism following on the earlier and more rapid exhaustion of the carbohydrate reserves, and that any slight increase in heat production is due to the specific dynamic action of the protein. Such may indeed be the true interpretation of the data, but in addition they seem to suggest that this temporary and early acceleration of the catabolism of protein and also probably fat is a special effort to meet an urgent need.

Summary.

1. In normally healing fracture cases the metabolism of calcium showed but little change. Intake on the average balanced output. There was, however, a marked loss of nitrogen, phosphorus and sulphur, the main excretory path for these catabolites being the kidneys. The rate of loss following the injury reached a maximum about the second to sixth day and then gradually declined. There was still a slight but continuous loss even after the lapse of 1 or 2 months.

2. The S : N and P_2O_5 : N ratios suggested that the material catabolised was probably mainly muscle.

3. In otherwise normal fracture patients, the administration of irradiated ergosterol did not appear to alter the metabolism apart from a slight increase in the urinary excretion of calcium; nor was there any special clinical improvement to be attributed to this form of therapy.

4. Metabolic analyses did not throw any light on the problem of the ununited fracture.

5. The beneficial effects of irradiated ergosterol in certain types of pathological fracture and abnormal bone conditions have been demonstrated.

6. A catabolic loss of sulphur, nitrogen and phosphorus resulting from the injury of tissue other than bone has been demonstrated.

7. Brief mention has been made of a few cases of abnormal bone metabolism in so far as they illustrate some of the phases of calcium and phosphorus metabolism.

8. There does not appear to be any coincident increase in the non-protein-nitrogen and inorganic phosphorus of the blood corresponding to the increased catabolism noted in cases of fracture and non-bony injury. Changes do occur later, which appear to involve a raising of the level of these elements.

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